REMARKS

The Examiner rejected claims 1-5 and 8-23 under 35 U.S.C. § 103(a) as allegedly being unpatentable over the combined teachings of Stacker et al. (J. Biol. Chem. 274:32127-32136, 1999, hereinafter "Stacker") and Joukov et al. (EMBO J., 16:3898-3911, 1997, hereinafter "Joukov"). Applicants request reconsideration of the rejection is view of the following remarks.

Joukov and Stacker disclose that VEGF-C and VEGF-D, respectively, are activated by proteolytic processing. Neither of these documents discloses nor suggests the use of plasmin for the activation of VEGF-C or VEGF-D.

The Examiner asserted that it would be obvious for one of skill in the art to contact the isolated VEGF-C or VEGF-D with plasmin in order to activate these molecules because plasmin is a natural protease which cleaves VEGF-A and there would be an expectation that plasmin would also be useful for cleaving VEGF-C and VEGF-D because of the structural similarities of the VEGF molecules. Applicants disagree.

The Examiner failed to set forth a *prima facie* case for obviousness because the Examiner failed to articulate exactly what "structural similarity" would have created the expectation that plasmin would have similar effects on different molecules.

The primary amino acid sequences are not sufficiently similar to warrant the Examiner's conclusion. In fact, the structural similarity between VEGF-A and VEGF-C or VEGF-D is quite low at the amino acid level, as shown by the alignment of the VEGF-A, VEGF-C and VEGF-D amino acid sequences set forth in Appendix A. VEGF-C and VEGF-D share less than approximately 30% identity with VEGF. One of ordinary skill in the art would not have expected that a protease that cleaved one protein in a specific way would cleave a protein with less than about 30% amino acid sequence identity in a similar way.

The prior art demonstrates that VEGF-C/D proteins were each understood to be structurally *dissimilar* to VEGF in other important ways, including the manner in which their genes are transcribed/translated; post-translational processing; the effects of processing on activity; dimerization; and heparin binding. The Joukov article cited by the PTO (for the teaching that plasmin cleaves high molecular weight forms of VEGF-A) cites articles by

Houck (1992) and (Keyt 1996) as support for its statements regarding plasmin. The Houck document (set forth in Appendix B) teaches or suggests that high molecular weight forms of VEGF-A bind to heparin-containing proteoglycans, and that plasmin digestion causes release of a dimeric VEGF-A species that is an endothelial mitogen. (See appendix and pp. 26034-35.) Houck teaches that the higher molecular weight species that bind heparin have a domain rich in basic residues, whereas the lower molecular weight forms that fail to bind heparin lack this domain. Keyt (set forth in Appendix C) further teaches that VEGF-A is expressed in a variety of tissues as at least 121, 165, 189, and 206 amino acid forms due to *alternative RNA splicing*. Keyt teaches that the endothelial mitogenic potencies of certain short forms lacking the heparin binding domain (VEGF-A110 and VEGF-A121) were **decreased** more than 100-fold compared to that of VEGF165, a heparin binding form. (See abstract.)

The Joukov article paints a very different picture of VEGF-C. Joukov does not teach that any alternative RNA splicing occurs with VEGF-C. Joukov explains that VEGF-C is originally expressed as a prepro-protein that is much larger, structurally, than the largest VEGF-A precursor. "The VEGF homology domain spans only about one-third of the cysteine-rich VEGF-C precursor." (Abstract.) VEGF-A does not have a cysteine-rich C-terminus analogous to the cysteine-rich C-terminus of prepro-VEGF-C. In contrast, VEGF-C has no reported heparin binding domain or heparin binding activity, whereas larger isoforms of VEGF-A do.

Joukov also teaches that different forms of VEGF-C were produced by proteolytic processing. "The stepwise proteolytic processing of VEGF-C generated several VEGF-C forms with increased activity towards VEGFR-3, but only the fully processed VEGF-C could activate VEGFR-2." As already noted above, Keyt teaches that proteolytic processing of VEGF-A appeared to **decrease** VEGF-A activity about 100-fold.

Joukov also teaches, "*Unlike other members of the PDGF/VEGF family*, mature VEGF-C formed mostly non-covalent homodimers." (Emphasis added.)

Stacker describes VEGF-D as having structural homology with VEGF-C (although they share only 61% amino acid sequence identity with each other in the "VEGF homology domain") and also as having the receptor specificity of VEGF-C. Stacker teaches

that the fully processed form of VEGF-D has ~290-fold greater affinity for VEGFR-2 and ~40-fold greater affinity for VEGFR-3 than unprocessed VEGF-D.

The molecules also exhibit structural dissimilarity with respect to receptors to which they bind. Activated forms of VEGF bind to VEGFR-1 and VEGFR-2, while activated forms of VEGF-C and VEGF-D bind to VEGFR-2 and VEGFR-3.

In view of the foregoing detailed analysis contrasting VEGF-A with VEGF-C/D, it should be clear that the vague premise of "structural similarities between the VEGF molecules" does not withstand scrutiny, and the Patent Office has failed to articulate a *prima facie* case of obviousness.

Even if one of ordinary skill in the art as of the filing date of this application would have expected that plasmin would successfully cleave VEGF-C or VEGF-D -- a proposition which the Applicants dispute -- there would have been no expectation that the cleavage of VEGF-C or VEGF-D by plasmin would result in an *active* VEGF-C or VEGF-D molecule at all, let alone an *activation effect* as recited in, e.g., claim 1. The combined teachings of the cited art fail to disclose or suggest that cleavage of VEGF-C or VEGF-D by plasmin would result in an active molecule. While Joukov reports that high molecular weight forms of VEGF-A can be cleaved by plasmin, it does not disclose or suggest that such cleavage would result in an *active* VEGF-A molecule (i.e., ability to binds VEGFR-1 or VEGFR-2). In fact, the Keyt reference, cited by Joukov, teaches 100-**reduction** in activity for shorter forms of VEGF-A, compared to unprocessed VEGF-A165. Keyt also ran experiments with numerous proteases, some of which failed to cleave VEGF-A and others of which (trypsin) generated a series of smaller fragments. Importantly, the cited art does not disclose or suggest that plasmin would cleave VEGF-C or VEGF-D at the processing site(s) necessary to produce an active molecule (i.e., ability to bind to VEGFR-2 and/or VEGFR-3), and cleave *only* at such processing sites, as opposed to other sites that would diminish or destroy activity. It should also be remembered that the field of the invention – biotechnology – is generally considered to be an unpredictable field, and unpredictability supports a conclusion of unobviousness.

Finally, because one of skill in the art would not expect that the cleavage of VEGF-C or VEGF-D by plasmin would result in active VEGF-C or active VEGF-D, there would be no expectation of success of arriving at the claimed invention.

To summarize, it would have been unpredictable that a protease which cleaved and diminished the activity of one protein (VEGF-A) could have been used to properly process, *and activate*, the activity of a structurally dissimilar protein (VEGF-C or VEGF-D). Thus, the Examiner has failed to establish a *prima facie* case of obviousness for the subject matter of any of claims 1-5 and 8-23 under 35 U.S.C. § 103(a) over Joukov in view of Stacker. Accordingly, the rejection should be withdrawn.

If the Examiner believes that a telephone conversation would expedite allowance of the claims, she is invited to contact the undersigned agent or David A. Gass, Attorney for Applicants, at the number below.

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Respectfully submitted,

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APPENDIX A

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CLUSTAL 2.0.5 multiple sequence alignment


VEGF-C    MHLLGFFSVACSLLAAALLPGPREAPAAAAAFESGLDLSDAEPDAGEATAYASKDLEEQL 60
VEGF-D    MYREWVVVNVFMMLYVQLVQGS----------------SNEHGPVKRSSQSTLERSEQQI 44
VEGF-A    -----------PSPSYHLLPGR----------------------RRTVDAAASRGQGPEP 27
                          *: *                                : .    :

VEGF-C    RSVSSVDELMTVLYPEYWKMYKCQLRKGGWQHNREQANLNSRTEETIKFAAAHYNTEILK 120
VEGF-D    RAASSLEELLRITHSEDWKLWRCRLRLKSFTS----MDSRSASHRSTRFAATFYDIETLK 100
VEGF-A    APGGGVEGVG--ARGVALKLFVQLLGCSRFGG-------------AVVRAGEAEPSGAAR 72
           . ..:: :          *::   *    :                :   *.        :

VEGF-C    SIDNEWRKTQCMPREVCIDVGKEFGVATNTFFKPPCVSVYRCGGCCNSEGLQCMNTSTSY 180
VEGF-D    VIDEEWQRTQCSPRETCVEVASELGKSTNTFFKPPCVNVFRCGGCCNEESLICMNTSTSY 160
VEGF-A    SASSGREEPQPEEGEEEEEKEEERG--------PQWRLGARKPGSWTGEAAVCADS---- 120
            ..  ...*    *   :  .* *        *       *  *. . *.  * ::

VEGF-C    LSKTLFEITVPLSQGPKPVTISFANHTSCRCMSKLDVYRQVHSIIRRSLPATLP-QCQAA 239
VEGF-D    ISKQLFEISVPLTSVPELVPVKVANHTGCKCLP--TAPRHPYSIIRRSIQIPEEDRCSHS 218
VEGF-A    ---------APAARAPQALARASGRGGRVARRGAEESGPPHSPSRRGSASRAGPGRASET 171
                    .* :  *: :.   ..                  .  * *   .   :.. :

VEGF-C    NKTCPTNYMWNNHICRCLAQEDFMFSSDAGDDSTDGFHDICGPNKELDEETCQCVCRAGL 299
VEGF-D    KKLCPIDMLWDSNKCKCVLQEENPLAGTEDHSHLQ------------------------- 253
VEGF-A    MNFLLSWVHWSLALLLYLHHAK-------------------------------------- 193
           :       *.      : : .

VEGF-C    RPASCGPHKELDRNSCQCVCKNKLFPSQCGANREFDENTCQCVCKRTCPRNQPLNPGKCA 359
VEGF-D    EPALCGPHMMFD-----------------------EDRCECVCKTPCPKDLIQHPKNCS 289
VEGF-A    ------------------------------------------------VSGRALLAPRAAA 206
                                                          .      *  .:

VEGF-C    C-ECTESPQKCLLKGKKFHHQTCSCYR------RPCTNRQKACEPGFSYSEEVCRCVPSY 412
VEGF-D    CFECKESLETCCQKHKLFHPDTCSCEDRCPFHTRPCASGKTACAKHCRFPKEKRAAQGPH 349
VEGF-A    SASPGWG-------------------------RACERARGGSVPHAGPWEKKKKKK--- 237
          . .   .                          *.*   : ..       ::

VEGF-C    WKRPQMS 419
VEGF-D    SRKNP-- 354
VEGF-A    -------
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APPENDIX B

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Dual Regulation of Vascular Endothelial Growth Factor Bioavailability by Genetic and Proteolytic Mechanisms*

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The vascular endothelial growth factor (VEGF) family encompasses four polypeptides that result from alternative splicing of mRNA. We have previously demonstrated differences in the secretion pattern of these polypeptides. Stable cell lines expressing VEGFs were established in human embryonic kidney CEN4 cells. $VEGF_{121}$, the shortest form, was secreted and freely soluble in tissue culture medium. $VEGF_{189}$ was secreted, but was almost entirely bound to the cell surface or extracellular matrix. $VEGF_{165}$ displayed an intermediary behavior. Suramin induced the release of $VEGF_{189}$, permitting its characterization as a more basic protein with higher affinity for heparin than $VEGF_{165}$ or $VEGF_{121}$, but with similar endothelial cell mitogenic activity. Heparin, heparan sulfate, and heparinase all induced the release of $VEGF_{165}$ and $VEGF_{189}$, suggesting heparin-containing proteoglycans as candidate VEGF-binding sites. Finally, $VEGF_{165}$ and $VEGF_{189}$ were released from their bound states by treatment with plasmin. The released 34-kDa dimeric species are active as endothelial cell mitogens and as vascular permeability agents. We conclude that the bioavailability of VEGF may be regulated at the genetic level by alternative splicing that determines whether VEGF will be soluble or incorporated into a biological reservoir and also through proteolysis following plasminogen activation.

The establishment of a vascular supply is an absolute requirement for a number of both normal and pathological processes including embryogenesis, follicular development, wound healing, and tumorigenesis (1). A variety of factors have been implicated in the control of angiogenesis (1). However, vascular endothelial growth factor (VEGF)[1] is unique among these angiogenic factors by virtue of its direct effect on endothelial cell mitogenesis combined with the fact that it is a secreted polypeptide (2). It is also specific toward its target, with only endothelial cells reportedly responsive to this factor (3). Furthermore, its binding sites *in vivo* are present in endothelial cells, but not in other cell types (4). Intriguingly, multiple molecular species of VEGF can be generated due to alternative splicing of its RNA transcribed from a single gene (5, 6). Such diversity in molecular structure suggests distinct roles for these VEGF variants.

VEGF was isolated as a heparin-binding secreted factor from bovine pituitary folliculo-stellate cells (3). The purified protein was a glycosylated homodimer of ~45,000 Da. It stimulated mitogenesis in cultured vascular endothelial cells with half-maximal stimulation at 100–150 pg/ml and also promoted angiogenesis in the chick chorioallantoic membrane (3, 7, 8). VEGF was also purified independently as a tumor-secreted factor that induced vascular permeability as measured in the Miles assay (see Refs. 9 and 10). The tyrosine kinase receptor *flt* has recently been described as a high affinity cell-surface receptor for VEGF (11).

Molecular cloning of the human cDNA for VEGF from a promyelocytic leukemia cell line (HL-60) library yielded not only the transcript encoding the 45-kDa VEGF species described above, but also two other transcripts encoding VEGF species with insertions or deletions in the cDNA occurring at a common site (7). All three transcripts encoded a 26-amino acid hydrophobic signal sequence and had identical mature amino termini. The transcript encoding the 45-kDa form, $VEGF_{165}$, is expected to generate a 165-amino acid peptide following signal peptide cleavage. Relative to $VEGF_{165}$, $VEGF_{121}$ has a 44-amino acid deletion between positions 116 and 159, and $VEGF_{189}$ has a 24-amino acid insertion at position 116. The gene structure for VEGF has been elucidated and confirms that alternative splicing of VEGF RNA can generate these different transcripts (5, 6). Analysis of a variety of human cDNA libraries by the polymerase chain reaction using primers that flank the insertion/deletion site indicated that $VEGF_{165}$ is the predominantly expressed form in most of the libraries examined, although multiple transcript types were usually detected. A fourth molecular species, $VEGF_{206}$, was also identified during this screening (5). It is identical to $VEGF_{189}$, but contained an additional 17 codons following the 24-codon insertion in $VEGF_{189}$. No intron separated these two coding regions, suggesting that the inclusion or exclusion of these 17 codons is determined by the definition of the 5′-splice donor site during RNA processing much like the alternative splicing mechanism that generates either *lyn*12 or, with the inclusion of an additional 21 amino acids, *lyn*11 (12).

To study the potential differences in biological function of the polypeptides encoded by these multiple transcripts of VEGF, we expressed each in the human embryonic kidney cell line 293 (5). Subsequent analysis of the expressed proteins indicated that the two shorter forms, $VEGF_{121}$ and $VEGF_{165}$,

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[1] The abbreviations used are: VEGF, vascular endothelial growth factor; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; bFGF, basic fibroblast growth factor.

were secreted endothelial cell mitogens that behaved similarly. However, the two longer forms, $VEGF_{189}$ and $VEGF_{206}$, while expressed by the transfected cells, were not found as freely soluble forms in the media; and thus, their bioactivity could not be readily assessed (5). We have extended our study of the behavior of this family of polypeptides and found that the longer forms are secreted, but are bound to the cell surface or extracellular matrix. A significant percentage of secreted $VEGF_{165}$, but not $VEGF_{121}$, is also bound extracellularly. A variety of compounds were identified that induced release of the bound forms, thus allowing their biochemical and biological characterization. In addition, we demonstrate potentially physiologically relevant proteolytic release mechanisms. These data suggest that the bioavailability of VEGF can be regulated both at the RNA level, through alternative splicing mechanisms, and at the protein level, through proteolysis.

EXPERIMENTAL PROCEDURES

Reagents—Suramin was obtained from Mobay Chemical Corp. (New York). Heparinase I (EC 4.2.2.7), heparinase III (heparin-sulfate lyase, EC.4.2.2.8), aprotinin, and Evans blue dye were from Sigma. Plasmin (18.6 casein units/mg) was purchased from AB Kabi (Stockholm, Sweden). Both phosphatidylinositol-specific phospholipase C and phosphatidylcholine-specific phospholipase C were from Boehringer Mannheim. Recombinant human $VEGF_{165}$ was purified to homogeneity from conditioned medium of transfected Chinese hamster ovary cells (13).

Establishment of Stable Cell Lines—The VEGF cDNA encoding each form of VEGF was subcloned from a pRK vector into mammalian expression vector pHEBO23 (14). To produce stable cell lines expressing VEGF, CEN4 cells were used. These cells are a derivative of the human embryonic kidney cell line 293 that stably expresses Epstein-Barr virus nuclear antigen-I, required for episomal replication of the pHEBO23 vector (15). CEN4 cells were removed with trypsin, and 3×10^6 cells were transfected with 5 μg of vector DNA by electroporation (16). A stable cell population was established by selection with 200 μg/ml hygromycin (Calbiochem). Since a previous study with the episomal pHEBO23 vector showed no difference in protein expression levels between cell lines derived from individual colonies and those derived by combining all resistant colonies in the plate, all resistant cells were combined after 2 weeks to create cell lines expressing each form of VEGF (14). The cell lines were maintained in Ham's F-12/DMEM (50:50; GIBCO/BRL) with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 1 mM glutamine, 200 μg/ml G418 (Geneticin, GIBCO/BRL) to maintain Epstein-Barr virus antigen-I expression, and 200 μg/ml hygromycin. Cells were passed ~1:10 every 3–4 days; no loss of protein expression was noted at up to 20 passages.

Cation-exchange and Heparin-Sepharose Affinity Chromatography—Media conditioned by stable cell lines expressing $VEGF_{121}$, $VEGF_{165}$, or $VEGF_{189}$ in the presence or absence of 1 mM suramin were concentrated ~4-fold by Centriprep 10 (Amicon Corp,, Danvers, MA). For cation-exchange chromatography, 2.5 ml of concentrated conditioned medium were equilibrated with 25 mM sodium phosphate, pH 6.0, by PD-10 columns. Media were applied to S-Sepharose fast flow columns (1 ml; Pharmacia LKB Biotechnology Inc.) that had been pre-equilibrated with 25 mM sodium phosphate, pH 6.0. The columns were washed with 4 ml of starting buffer and then stepwise-eluted with the same buffer containing 0.2, 0.5, or 1.0 M NaCl. One-ml fractions were collected, and aliquots were subjected to VEGF ELISA. For heparin-Sepharose affinity chromatography, either concentrated conditioned medium or peak S-Sepharose fractions were equilibrated with 10 mM Tris/HCl, pH 7.2, containing 50 mM NaCl. The material was loaded onto 1-ml columns that had been equilibrated with the same buffer. After washing, the column was stepwise-eluted with 10 mM Tris/HCl, pH 7.2, containing 0.15, 0.9, 2.0, or 3.0 M NaCl. One-ml fractions were collected and assayed for VEGF content.

Metabolic Labeling and Immunoprecipitation—Cells were trypsinized and plated at 5×10^5 cells/well in 35-mm dishes (Becton-Dickinson, Lincoln Park, IL). After 36 h, cells were incubated for 30 min in DMEM lacking cysteine and methionine at 37 °C. The medium was then switched to DMEM lacking cysteine and methionine, but containing a 100 μCi/ml concentration each of L-[^{35}S]cysteine and L-[^{35}S]methionine for 2 h. Finally, the cells were incubated for 6 h in serum-free complete medium (PSO4). The tissue culture medium was harvested, cleared by centrifugation, and immunoprecipitated with 10 μg of purified monoclonal antibody (mAb) A4.6.1 (17) per 500 μl of medium for 2 h on ice, followed by the addition of 5 μl of rabbit anti-mouse antiserum (Organon-Technika, Durham, NC) for 1 h on ice. The immune complexes were precipitated with 50 μl of protein A-Sepharose (Pharmacia), washed with radioimmune precipitation buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0), extracted with 50 μl of SDS-polyacrylamide gel sample buffer, and analyzed by SDS-PAGE (Novex, San Diego, CA). Gels were fixed, treated with AMPLIFY (Amersham Corp.), dried, and exposed to x-ray film.

Endothelial Cell Growth Assay—Bovine capillary endothelial cells were maintained in low glucose DMEM containing 10% calf serum as previously described (3). For cell proliferation assays, cells were plated at 8×10^3 cells/well in 12-well plates in DMEM supplemented with 10% calf serum, 2 mM glutamine, and antibiotics (assay volume, 2 ml). Various aliquots of conditioned medium from CEN4 cell lines stably expressing the different forms of VEGF were added, and cell number was determined after 5 days by a Coulter Counter following dissociation by trypsin. When mAb A4.6.1, which neutralizes the bioactivity of VEGF (17), was used, it was included at 800 ng/ml at the time of the addition of conditioned medium.

VEGF ELISA—Ninety-six-well microtiter plates (Maxisorb, Nunc, Kamstrup, Denmark) were coated with mAb A4.6.1 (17) by incubation overnight at 4 °C with 100 μl of antibody/well at 2.5 μg/ml in 50 mM sodium carbonate, pH 9.6 (coat buffer). After removal of the coat buffer, the coated plates were blocked with a 150 μl/well concentration of 5 mg/ml bovine serum albumin in phosphate-buffered saline for 1 h at room temperature and washed six times with 0.5 mg/ml Tween 20 in phosphate-buffered saline (wash buffer).

Standards were freshly prepared by dilution of recombinant human $VEGF_{165}$ with assay buffer (phosphate-buffered saline containing 5 mg/ml bovine serum albumin, 0.05% Tween 20, and 0.01% thimerosal). The diluted standard and samples were dispensed onto the coated plates (100 μl/well). Plates were sealed and incubated at room temperature for 2 h with gentle agitation. Wells then were washed six times with wash buffer. Monoclonal antibody 3.13.1 (17) or rabbit anti-human VEGF polyclonal antiserum was conjugated with horseradish peroxidase and added at 100 μl/well (18). To measure $VEGF_{121}$, the polyclonal antiserum was used since mAb 3.13.1 does not recognize this species of VEGF (17). Plates were incubated for 2 h at room temperature and washed six times with wash buffer, and a 100 μl/well concentration of substrate solution was added (0.4 g of *o*-phenylenediamine dihydrochloride in 1 liter of phosphate-buffered saline plus 0.4 ml of 30% hydrogen peroxide; Sigma). Plates were incubated in the dark for 15 min and stopped by the addition of 100 μl of 2.25 M sulfuric acid, followed by determination of the absorbance at 490 nm on a V_{max} plate reader (Molecular Devices, Menlo Park, CA). A standard curve was generated by plotting absorbance *versus* log of recombinant human $VEGF_{165}$ concentration using a four-parameter nonlinear regression curve-fitting program. Sample concentrations were obtained by interpolation of their absorbance on the standard curve.

Miles Vascular Permeability Assay—Induction of vascular permeability was determined using the Miles assay (19). Briefly, anesthetized guinea pigs were injected via the tail vein with 1 ml of 0.5% (w/v) Evans blue dye. Thirty min later, 200 μl of conditioned medium from the CEN4 cell lines were injected intradermally into the back of the guinea pig. Leakage of dye bound to serum proteins was detected by the presence of an intense blue spot surrounding the injection site.

RESULTS

Establishment of Stable VEGF Cell Lines—To analyze the individual molecular species of VEGF, stable mammalian cell lines for each were established. Fig. 1*A* illustrates the primary amino acid sequence for three molecular species of VEGF, with the *unboxed* residues common to all forms of VEGF (*i.e.* the entire sequence for $VEGF_{121}$), the 44 amino acids found only in $VEGF_{189}$ indicated by the *dashed underline*, and the 24 amino acids found in $VEGF_{165}$ and $VEGF_{189}$ indicated by the *solid underline*. The additional 17 amino acids in $VEGF_{206}$ are not shown. Since $VEGF_{206}$ behaves very similarly to

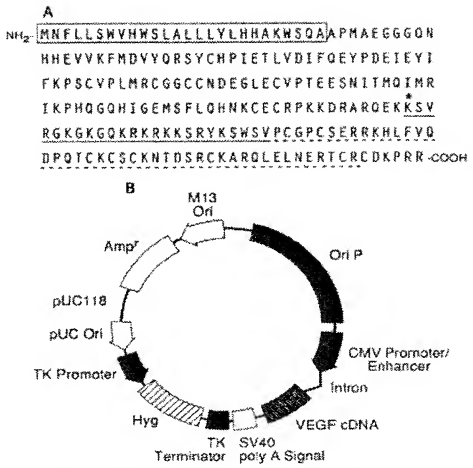

FIG. 1. **Amino acid sequence of VEGF and expression vector used for creating VEGF-expressing cell lines.** *A*, composite amino acid sequence for $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$. *Boxed* residues represent the 26-amino acid hydrophobic signal peptide. The *dashed underline* indicates residues found only in $VEGF_{189}$; the *solid underline* designates those in both $VEGF_{189}$ and $VEGF_{165}$; and the *unboxed* residues are in all three forms. The *asterisk* indicates the lysine in $VEGF_{189}$ and $VEGF_{121}$ that is changed to a glutamine in $VEGF_{165}$. *B*, the episomal plasmid pHEBO23, which was used to create stable CEN4 cell lines expressing each form of VEGF. Expression of the VEGF cDNA was driven by the cytomegalovirus (*CMV*) promoter/enhancer. The plasmid was maintained by the hygromycin phosphotransferase gene of *Escherichia coli* (*Hyg*), which confers resistance to hygromycin B in mammalian cells. The origin of replication (*OriP*) was derived from Epstein-Barr virus. Expression of the Hyg gene was driven by the thymidine kinase (TK) promoter.

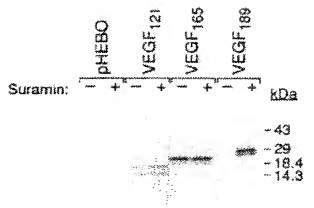

FIG. 2. **Effect of suramin on release of VEGF.** Stable CEN4 cell lines expressing each form of VEGF or the vector control cell line was metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine as described under "Experimental Procedures." Suramin was included at 1 mM where indicated for a 6-h chase period in serum-free medium. VEGF was immunoprecipitated with mAb A4.6.1 and analyzed by SDS-PAGE under reducing conditions, followed by autoradiography.

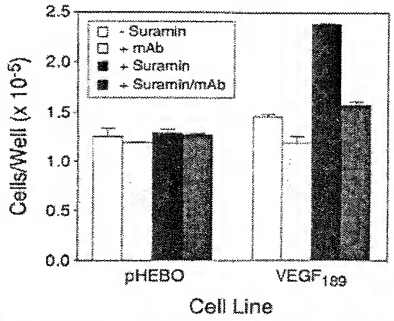

FIG. 3. **Bioactivity of $VEGF_{189}$.** The cell line expressing $VEGF_{189}$ and the control cell line, pHEBO, were used to condition serum-free media for 24 h in the absence or presence of 1 mM suramin. The conditioned media were dialyzed extensively to remove the suramin and tested for mitogenic activity toward bovine capillary endothelial cells as described under "Experimental Procedures." The *bars* represent the average of triplicate determinations obtained from a single dose of conditioned medium (50 μl/well). Where indicated, neutralizing mAb A4.6.1 was included at 800 ng/ml in the assay.

$VEGF_{189}$ in all parameters examined in this study, only data obtained with $VEGF_{189}$ will be shown. All three forms have identical 26-amino acid hydrophobic signal peptides as specified by the *boxed* residues. The cDNAs encoding each of these sequences were cloned into pHEBO23, the Epstein-Barr virus-derived vector shown in Fig. 1*B* (13). Expression of the cDNAs was driven by the human cytomegalovirus promoter. This vector replicates episomally in the nuclei of the cell line CEN4, a human embryonic kidney 293 cell line derivative that stably expresses the Epstein-Barr virus nuclear antigen-I (14). Following transfection of CEN4 cells by electroporation of plasmid DNA (16), stable populations were generated by selection with 200 μg/ml hygromycin.

Release of $VEGF_{189}$ by Suramin—We had previously shown that $VEGF_{189}$ was produced by cells transfected with its cDNA, but that despite the presence of a hydrophobic signal peptide, little or no $VEGF_{189}$ was found in a freely soluble form in the tissue culture medium (5). Because it was possible that the secreted polypeptide was bound to an extracellular receptor of some type, we tested suramin, a polyanionic compound known to interfere with the binding of a variety of growth factors to receptors (20–22), for its ability to release $VEGF_{189}$ from its extracellular binding site. Cell lines were labeled with [^{35}S]cysteine and [^{35}S]methionine for 2 h, followed by a 6-h chase in serum-free medium in the absence or presence of 1 mM suramin. Aliquots of conditioned medium were immunoprecipitated using anti-VEGF mAb A4.6.1 (17). SDS-PAGE analysis of the immunoprecipitated products indicated that $VEGF_{165}$ was present in the conditioned medium, but that little or no $VEGF_{189}$ of the mature size could be detected in the absence of suramin (Fig. 2). With suramin treatment, however, bands of ~26,000 and 24,000 Da under reducing conditions were detected. The doublets for all species of VEGF seen on polyacrylamide gels result from glycosylation of VEGF; immunoprecipitations from cells labeled in the presence of tunicamycin showed only the lower band (data not shown). To determine the bioactivity of $VEGF_{189}$, the conditioned medium was dialyzed to remove the suramin and tested in an endothelial cell growth assay. Fig. 3 demonstrates that $VEGF_{189}$ released by suramin stimulated endothelial cell growth. This mitogenic response was ~60% of that stimulated by 1 ng/ml bFGF under identical conditions. Higher doses of conditioned medium became progressively inhibitory due to the inability to completely remove the suramin. Little or no activity was detected in the conditioned medium from the control cell line, pHEBO. To clearly prove that the released activity resulted from VEGF, neutralizing mAb A4.6.1 was included in the assay, and it inhibited virtually all the suramin-released mitogenic stimulus.

Biochemical Characterization of VEGF—Previous reports on the biochemical nature of VEGF examined the 165-amino acid form of the polypeptide and showed it to be a heparin-binding basic protein with an isoelectric point of ~8.5 (3, 8). To gain insight into some of the biochemical characteristics of $VEGF_{121}$ or $VEGF_{189}$, we compared the chromatographic behavior displayed by these molecular forms on S-Sepharose and heparin-Sepharose columns to that exibited by $VEGF_{165}$. The proteins were from the conditioned media of stably transfected CEN4 cell lines. Whereas $VEGF_{165}$ bound fairly tightly to S-Sepharose at pH 6.0 and was completely eluted

in the presence of 0.5 M NaCl, $VEGF_{189}$ bound much more strongly and was eluted only with 1.0 M NaCl (Fig. 4), similar to bFGF (23). On the other hand, $VEGF_{121}$ has very weak affinity for S-Sepharose, but binds strongly to an anion-exchange column such as Q-Sepharose (data not shown). $VEGF_{121}$ failed to bind to heparin-Sepharose. $VEGF_{165}$ bound strongly, but was completely eluted in the presence of 0.9 M NaCl, in agreement with previous studies (3). $VEGF_{189}$ bound and was only 20–30% eluted in the presence of 0.9 M NaCl. The remainder of the bound material was removed with 2 M NaCl. This chromatographic profile is also similar to that exhibited by bFGF (23). The chromatographic behavior of the different VEGF forms is consistent with their primary sequence: compared to $VEGF_{121}$, the 44 additional amino acids in $VEGF_{165}$ contain many basic residues. Remarkably, the additional 24 amino acids of $VEGF_{189}$ are comprised of 12 basic residues. Thus, alternative splicing of VEGF is responsible for turning a weakly acidic polypeptide, $VEGF_{121}$, into increasingly more basic heparin-binding ones.

Effect of Heparin and Heparinase on VEGF Release—The finding that a strongly basic $VEGF_{189}$ was bound to an unknown extracellular site suggested similarity to the behavior of bFGF and the B-chain of platelet-derived growth factor (24–26). These molecules are known to bind to heparin-containing compounds in the extracellular matrix or on the cell surface. We thus examined the ability of soluble heparin or heparan sulfate to compete for binding with $VEGF_{189}$ to the extracellular site. The stable CEN4 cell line expressing $VEGF_{189}$ was metabolically labeled with ^{35}S-labeled amino acids as previously described, followed by a 6-h chase in serum-free PSO4 in the absence or presence of heparin or heparan sulfate. Immunoprecipitation of VEGF from the medium with mAb A4.6.1 followed by SDS-PAGE and autoradiography showed that heparin (at 100 μg/ml) induced the release of $VEGF_{189}$ into the cell culture medium (Fig. 5). Heparan sulfate did likewise (data not shown). As further evidence that $VEGF_{189}$ is bound to a heparin-containing extracellular site, metabolically labeled cell cultures were treated with heparinase I or III (heparatinase I) during the 6-h chase. Both enzymes induced the release of mature $VEGF_{189}$, suggesting heparin-containing proteoglycans as likely binding sites for $VEGF_{189}$ in these cell cultures.

Because the metabolic labeling, immunoprecipitation, and SDS-PAGE analysis were not quantitative, we measured the amount of VEGF released by heparin from individual CEN4 cell lines by ELISA. Heparin induced a dramatic increase in the release of $VEGF_{189}$ from virtually undetectable levels to values comparable to those measured in the media conditioned by cells expressing $VEGF_{165}$. The increase was dose-dependent, with a half-maximal effect at ~10 μg/ml. In contrast, heparin had no effect on the amount of $VEGF_{121}$ released into the medium. Somewhat surprisingly, the amount of $VEGF_{165}$ released in the presence of heparin increased 2–4-fold. Fig. 6 illustrates a representative experiment. Thus, the multiple molecular species of VEGF display three different behaviors as secreted factors: $VEGF_{121}$ is entirely soluble; $VEGF_{189}$ is virtually all bound to putative heparin-containing sites; and $VEGF_{165}$ is intermediary, with 50–70% bound.

Plasmin Release of VEGF—Whereas suramin and heparin served to define the extracellular binding capacity of the longer forms of VEGF, they would not represent physiological release mechanisms for these species. To test potentially relevant means of release, several enzymatic treatments were carried out on metabolically labeled cultures of the stable VEGF cell lines. bFGF is known to bind to a phosphatidylinositol-anchored heparan sulfate proteoglycan in human bone marrow cultures and can be released as a bFGF-heparan sulfate proteoglycan complex by digestion cleavage with phosphatidylinositol-specific phospholipase C (27). We attempted the same treatment on metabolically labeled CEN4 cultures, followed by immunoprecipitation and SDS-PAGE. Neither phosphatidylinositol-specific phospholipase C nor phosphatidylcholine-specific phospholipase C treatment of $VEGF_{189}$ was effective in releasing this long form of VEGF (data not

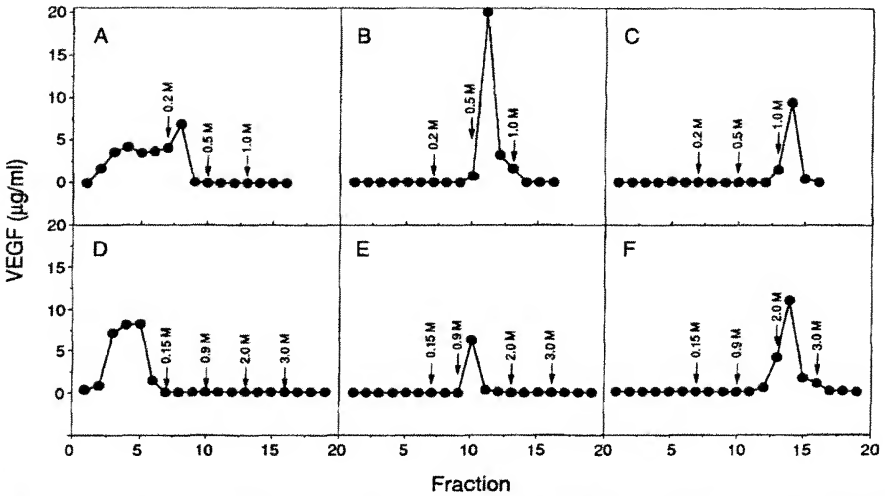

FIG. 4. **Chromatographic behavior of $VEGF_{121}$ (*A* and *D*), $VEGF_{165}$ (*B* and *E*), or $VEGF_{189}$ (*C* and *F*) on S-Sepharose (*A–C*) or heparin-Sepharose (*D–F*) columns.** VEGF levels were assessed by ELISA. In *A–C*, conditioned medium from transfected cells was concentrated and equilibrated in 25 mM sodium phosphate, pH 6.0. The material was loaded onto the columns and eluted in the presence of increasing concentrations of NaCl. Data shown for $VEGF_{189}$ chromatography were obtained following suramin treatment of the cell cultures. In *D*, conditioned medium from cells expressing $VEGF_{121}$ was equilibrated with 10 mM Tris/HCl, pH 7.2, containing 50 mM NaCl and loaded onto a heparin-Sepharose column. The column was eluted in the presence of increasing NaCl concentrations, as indicated. In *E* and *F*, peak S-Sepharose fractions were used since the residual of suramin significantly interfered with binding to heparin-Sepharose.

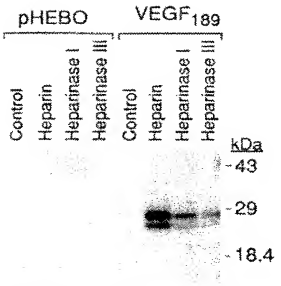

FIG. 5. **Release of $VEGF_{189}$ by heparin and heparinase.** The control cell line, pHEBO, or the $VEGF_{189}$ cell line was metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine as described under "Experimental Procedures." Heparin was included at 100 μg/ml during the 6-h chase period where indicated. Heparinase I or III (heparatinase) was used at 10 IU/ml. VEGF was immunoprecipitated with mAb A4.6.1 and analyzed under reducing conditions by SDS-PAGE.

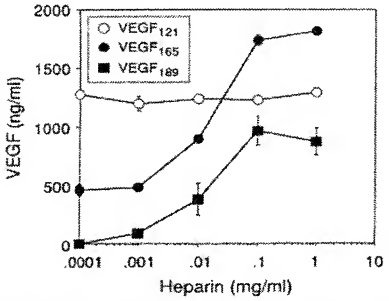

FIG. 6. **Quantitation of bound VEGF.** Each VEGF-expressing cell line was treated for 24 h in serum-free medium with increasing amounts of heparin. The amount of VEGF present in the conditioned medium was quantitated by ELISA as described under "Experimental Procedures." Points represent the mean of triplicate determinations from a representative experiment. No VEGF could be detected in medium conditioned by the control cell line, pHEBO (data not shown).

shown). However, a 20-min digestion with the serine protease plasmin induced the release of an ~17-kDa form and a 15-kDa form of $VEGF_{189}$ as well as peptides of the identical size from the $VEGF_{165}$ cell line as detected by immunoprecipitation and SDS-PAGE analysis under reducing conditions (Fig. 7, *upper*). These peptides were capable of dimerization as evidenced by the 34- and 30-kDa forms seen under nonreducing conditions (Fig. 7, *lower*). Following inhibition of protease activity with aprotinin, the conditioned media from these cell lines were tested for activity in the endothelial cell proliferation assay. Both were active as endothelial cell mitogens, whereas no activity could be detected in the medium conditioned for 20 min without plasmin (Fig. 8). The plasmin-released VEGF species did not bind to heparin, analogous to $VEGF_{121}$ (data not shown). In addition, these conditioned media were tested for induction of vascular permeability in the Miles assay (19). For this assay, serum-free medium was conditioned for 6 h by the stable cell lines, and plasmin was added at 0.1 CU/ml directly to this medium for the last 20 min at 37 °C. Following neutralization of the plasmin activity, media conditioned by the $VEGF_{165}$ cell line in the absence or presence of plasmin and by the $VEGF_{189}$ cell line only in the presence of plasmin stimulated significant leakage of Evans blue dye from vessels in guinea pig skin (Fig. 9). Media from the control cell line, pHEBO, and the $VEGF_{189}$ cell line in the absence of plasmin were inactive.

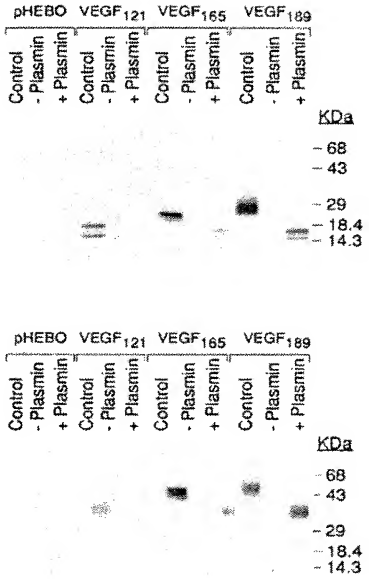

FIG. 7. **Proteolytic release of VEGF.** Cell lines expressing each form of VEGF were metabolically labeled with [^{35}S]cysteine and [^{35}S] methionine as described under "Experimental Procedures." Following a 6-h chase period in serum-free medium, the cultures were washed with fresh medium, and an additional incubation for 20 min at 37 °C in the absence or presence of 0.1 casein units/ml plasmin was carried out. Following neutralization of plasmin activity with aprotinin, VEGF was immunoprecipitated from these 20-min incubations as well as from the 6-h chase with mAb A4.6.1. Samples were analyzed by SDS-PAGE under reducing (*upper*) and nonreducing (*lower*) conditions, followed by autoradiography. *Control* indicates the immunoprecipitated products from the 6-h chase period. For $VEGF_{189}$, heparin (100 μg/ml) was included in the serum-free chase medium; cultures not treated with heparin were used for plasmin digestion. The immunoprecipitated products from the 20-min incubation are labeled as *−Plasmin* and *+Plasmin*.

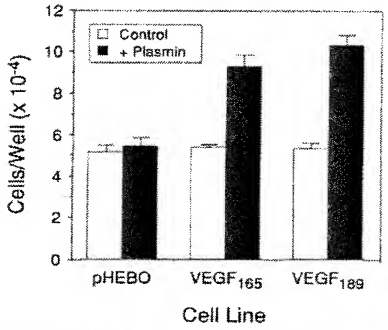

FIG. 8. **Mitogenic activity of proteolytically released VEGF.** Cell lines expressing $VEGF_{165}$ or $VEGF_{189}$ or the control cell line was plated at equal densities and 24 h later incubated for 6 h in serum-free medium. Cultures were washed with fresh medium and incubated for 20 min at 37 °C in the absence or presence of 0.1 CU/ml plasmin. Following neutralization of plasmin activity with aprotinin, the conditioned media were tested for mitogenic activity toward bovine capillary endothelial cells as described under "Experimental Procedures." The *bars* represent the average of triplicate determinations from a single dose of conditioned medium (100 μl) from a representative experiment.

DISCUSSION

Numerous growth factors and their receptors as well as

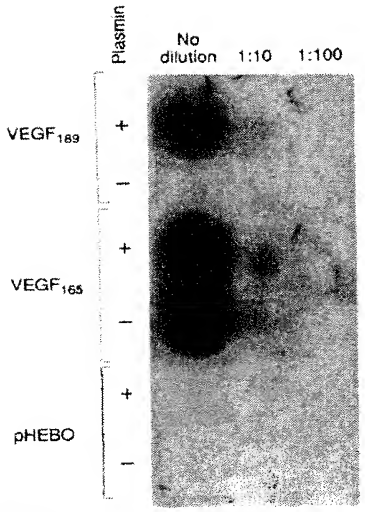

FIG. 9. **Vascular permeability activity of proteolytically released VEGF.** Cell lines expressing $VEGF_{165}$ or $VEGF_{189}$ or the control cell line was used to condition serum-free media for 6 h at 37 °C. During the last 20 min of this incubation, plasmin was added at 0.1 CU/ml where indicated. Plasmin activity was neutralized with aprotinin, and the media were tested at the indicated dilution for vascular permeability activity in the Miles assay (19) as described under "Experimental Procedures."

other proteins are known to exist in multiple forms as a result of alternative splicing of their RNAs. It has recently become evident that these multiple molecular species often exhibit significantly different biological behavior (26, 28, 29). One such family of polypeptides resulting from alternative splicing of RNA is the angiogenic direct-acting endothelial cell mitogen VEGF (5, 6). In this report, we demonstrated that the information supplied by alternative splicing has profound effects on the behavior of the translated proteins following secretion from the cell. The shortest form, $VEGF_{121}$, which is a 34–36-kDa homodimeric polypeptide, is secreted and freely soluble in the conditioned medium of the human embryonic kidney cell line CEN4 stably transfected with a $VEGF_{121}$ expression vector. The transcript of $VEGF_{165}$, the most common form of VEGF, contains an additional 44 codons relative to $VEGF_{121}$, resulting in a homodimeric protein of ~45 kDa. These 44 amino acids in the carboxyl-terminal end of the protein convert it to a basic polypeptide with heparin binding capability. In a stably transfected CEN4 cell line, $VEGF_{165}$ is secreted, but ~50–70% of the material binds to as yet unidentified components on the cell surface or in the extracellular matrix. $VEGF_{189}$, which contains an additional 24 amino acids highly enriched in basic residues, binds much more tightly to cation-exchange columns and to heparin-Sepharose. Little or no $VEGF_{189}$ is found in a freely soluble form in the conditioned medium of stably transfected CEN4 cells. Release of the bound forms of $VEGF_{165}$ and $VEGF_{189}$ by treatment with heparin, heparan sulfate, or heparinase suggests that the unknown binding site in these cell cultures involves a heparin-containing proteoglycan (30, 31).

A variety of recent studies have demonstrated that heparin sulfate-containing proteoglycans are the constituents of the extracellular matrix that are responsible for binding and concentrating a variety of growth factors in the matrix and at the cell surface. The most studied system of proteoglycan regulation of growth factor availability is that of bFGF. The binding of bFGF to heparin or heparan sulfate, either as soluble components of the tissue culture medium or as matrix- or cell surface-bound proteoglycans, appears to be necessary for bFGF binding to the high affinity FGF receptor (32). This requirement has not been completely understood. It may involve protection from degradation of FGF (33, 34); alteration of the conformation of FGF to that required for receptor binding (32, 35); or oligomerization of FGF in a manner facilitating dimerization of FGF receptors (36), a necessary step in signal transduction by all tyrosine kinase receptors examined thus far (37). The extracellular matrix may also serve as a reservoir for FGF since active bFGF-glycosaminoglycan complexes can be generated by proteolysis of the proteoglycan core protein (31) or by cleavage of a glycosylphosphatidylinositol membrane anchor by phospholipase C (27, 38). A variety of other growth factors also bind heparin or heparan sulfate: interleukin-3 and granulocyte-macrophage colony-stimulating factor (39), pleiotropin (40), heregulin (41), and hepatocyte growth factor (42). Recently, evidence has been provided for the involvement of cell surface-associated heparin-like molecules in enhancing binding of VEGF to high affinity receptors on endothelial cells in a manner similar to that reported for bFGF (43).

Whereas the CEN4 cell line is not known to endogenously express VEGF, heparin-binding sites are an integral component of many extracellular matrices. Thus, whatever cell type expresses VEGF, the described behavior of the different forms of VEGF could be expected. The source of VEGF secretion *in vivo* has not yet been thoroughly examined. Cultured aortic smooth muscle cells have been demonstrated to secrete VEGF and would represent a potential source capable of delivering VEGF directly to the endothelium *in vivo* (6, 44). *In situ* hybridization experiments demonstrated expression of the RNA transcript for VEGF in adult rat in a variety of well-vascularized organs such as pituitary, brain, heart, kidney, and lung (45, 46). Despite this expression, the endothelium is essentially quiescent in such organs, suggesting the VEGF produced does not actively function as a growth factor in this context. Perhaps by binding to the extracellular matrix, $VEGF_{189}$ and, to a significant extent, also $VEGF_{165}$ are sequestered in a reservoir providing ready access to angiogenic factors, as has been postulated for bFGF (47). Although cell surface-associated heparin-like molecules are thought to be necessary for VEGF binding to its receptor on endothelial cells and such a complex is an active endothelial cell mitogen (43), if the heparin-binding sites are not contiguous with endothelial cells, then it would be unlikely that the bound VEGF could activate those receptors. During periods of active angiogenesis, the freely soluble $VEGF_{121}$ may be especially important. In this context, it is interesting that this form of VEGF appears to be the predominant one found in placenta, an organ with very active angiogenesis (5). In the developing corpus luteum, another tissue undergoing angiogenesis, *in situ* hybridization demonstrated high levels of VEGF RNA expression, although the specific VEGF molecular species produced was not identified (48).

Binding of the long forms of VEGF to heparan sulfate proteoglycans in the extracellular matrix could provide a reservoir of biologically active VEGF available to endothelial cells following its release. We have demonstrated here that the serine protease plasmin induces the release of proteolytically clipped VEGF species of both $VEGF_{165}$ and $VEGF_{189}$ that are freely soluble in the tissue culture medium and are biologically active both as endothelial cell mitogens and as vascular permeability-enhancing agents. The size of the monomeric subunits as analyzed under reducing conditions by SDS-PAGE (~17 kDa) is very similar to that of the $VEGF_{121}$

monomer. In addition, mAb 3.13.1 does not immunoprecipitate the plasmin-cleaved forms of $VEGF_{165}$ and $VEGF_{189}$,[2] and it does not recognize $VEGF_{121}$ (17). Since $VEGF_{121}$ lacks 44 amino acids in the carboxyl terminus of the protein relative to $VEGF_{165}$, this suggests that the plasmin-released species of VEGF resulted from truncation of the proteins in the carboxyl termini, thus eliminating putative heparin-binding sites responsible for sequestration of the proteins in the extracellular matrix or on the plasma membrane.

Extensive studies have demonstrated the integral role of proteases during angiogenesis. The traditional paradigm for angiogenesis suggests that new capillary formation results from a cascade of processes beginning with degradation of the extracellular matrix of a venule following protease release or activation. This is followed by endothelial cell proliferation and migration, processes that also involve protease action through the degradation of the stroma of the tissue undergoing vascularization. Finally, the capillary lumen is formed; and again, proteases may be required (49). A potential key protease involved in these processes is plasmin (50–52). The active serine protease plasmin is generated by plasminogen activator cleavage of proteolytically inactive plasminogen. Plasminogen is present in serum and is relatively abundant in most tissues. Plasminogen activators of either or both the tissue and urokinase type are secreted from endothelial cells following stimulation with both VEGF and bFGF (53, 54). Therefore, secretion of plasminogen activators by endothelial cells in response to angiogenic agents would result in locally high concentrations of plasmin. In addition to previously demonstrated involvement in degradation of the extracellular matrix, both directly through digestion of such components of the basement membrane as fibronectin and laminin and indirectly by activating collagenases from their zymogens (55), plasmin may thus also serve to liberate angiogenic agents including $VEGF_{165}$ and $VEGF_{189}$ as well as bFGF from the extracellular matrix. It is possible that the spatial distribution of these angiogenic factors sequestered in the extracellular matrix may serve to orient vessel formation during angiogenesis by providing a network of positive growth stimuli.

In conclusion, several different molecular species of VEGF can be generated as a result of alternative splicing of VEGF RNA. The information encoded by this alternative splicing determines the fate of VEGF to be freely soluble or bound to the extracellular matrix or plasma membrane proteins, or properties of both. The proteolytic cascade of plasminogen activation, a key step during angiogenesis, can cleave the bound forms of VEGF, releasing a soluble factor capable of stimulating endothelial cell growth. Examining the nature of growth factors bound to extracellular matrix components *in vivo* may provide necessary information in understanding physiological cell growth regulation.

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APPENDIX C

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The Carboxyl-terminal Domain (111–165) of Vascular Endothelial Growth Factor Is Critical for Its Mitogenic Potency*

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Vascular endothelial growth factor (VEGF) is a potent and specific mitogen for endothelial cells. VEGF is synthesized and secreted by many differentiated cells in response to a variety of stimuli including hypoxia. VEGF is expressed in a variety of tissues as multiple homodimeric forms (121, 165, 189, and 206 amino acids/monomer) resulting from alternative RNA splicing. $VEGF_{121}$ is a soluble mitogen that does not bind heparin; the longer forms of VEGF bind heparin with progressively higher affinity. The higher molecular weight forms of VEGF can be cleaved by plasmin to release a diffusible form(s) of VEGF. We characterized the proteolysis of VEGF by plasmin and other proteases. Thrombin, elastase, and collagenase did not cleave VEGF, whereas trypsin generated a series of smaller fragments. The isolated plasmin fragments of VEGF were compared with respect to heparin binding, interaction with soluble VEGF receptors, and ability to promote endothelial cell mitogenesis. Plasmin yields two fragments of VEGF as indicated by reverse phase high performance liquid chromatography and SDS-polyacrylamide gel electrophoresis: an amino-terminal homodimeric protein containing receptor binding determinants and a carboxyl-terminal polypeptide which bound heparin. Amino-terminal sequencing of the carboxyl-terminal peptide identified the plasmin cleavage site as Arg^{110}-Ala^{111}. A heterodimeric form of $VEGF_{165/110}$, was isolated from partial plasmin digests of $VEGF_{165}$. The carboxyl-terminal polypeptide (111–165) displayed no affinity for soluble kinase domain region (KDR) or Fms-like tyrosine kinase (FLT-1) receptors. The various isoforms of VEGF (165, 165/110, 110, and 121) bound soluble kinase domain region receptor with similar affinity (approximately 30 pM). In contrast, soluble FLT-1 receptor differentiated VEGF isoforms (165, 165/110, 110, and 121) with apparent affinities of 10, 30, 120, and 200 pM, respectively. Endothelial cell mitogenic potencies of $VEGF_{110}$ and $VEGF_{121}$ were decreased more than 100-fold compared to that of $VEGF_{165}$. The present findings indicate that removal of the carboxyl-terminal domain, whether it is due to alternative splicing of mRNA or to proteolysis, is associated with a significant loss in bioactivity.

Angiogenesis, the growth of new vessels, is required for a wide variety of physiologic and pathologic proliferative processes. Recent evidence implicates vascular endothelial growth factor (VEGF),[1] an endothelial-cell specific mitogen and angiogenesis inducer (1, 2), as a critical regulator of normal and pathologic angiogenesis (3). VEGF mRNA expression is temporally and spatially related to proliferation of blood vessels in the ovarian corpus luteum or in the developing embryo (4, 5). Furthermore, recent studies have shown that monoclonal antibodies specific for VEGF are able to suppress the growth of human tumor cell lines in nude mice, suggesting that VEGF is an important mediator of tumor angiogenesis (6). VEGF mRNA undergoes alternative splicing events that lead to the production of four mature homodimeric proteins, each monomer having 121, 165, 189, or 206 amino acids ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, respectively) (7–9). $VEGF_{121}$ does not bind to heparin; in contrast, $VEGF_{165}$ and $VEGF_{189}$ bind to heparin with increasingly greater affinity. Cells transfected with cDNA encoding $VEGF_{121}$ or $VEGF_{165}$ secrete bioactive VEGF into the medium. In contrast, when $VEGF_{189}$ and $VEGF_{206}$ were expressed in mammalian cells, little or no VEGF can be found in the medium (9). In previous studies, we demonstrated that $VEGF_{189}$ or $VEGF_{206}$ are secreted but are almost completely bound to heparan sulfate containing proteoglycans in the extracellular matrix (10, 11). However, a diffusible fragment having qualitatively the same activity as intact VEGF could be released by plasmin. Extracellular matrix derived from cells expressing $VEGF_{189}$ or $VEGF_{206}$ and, to a lesser extent, $VEGF_{165}$, promotes the growth of vascular endothelial cells, demonstrating that matrix-bound VEGF is bioactive (11).

In the present study, we studied the interaction with heparin to address the biologic significance of the larger molecular forms of VEGF. We isolated and characterized plasmin-generated fragments of $VEGF_{165}$ and compared these fragments to native $VEGF_{165}$ or $VEGF_{121}$ with respect to various biochemical and biological functions. Our studies demonstrate that loss of the carboxyl-terminal domain, whether due to proteolysis or alternative splicing, correlates with a substantial decrease in endothelial cell mitogenic activity of VEGF.

EXPERIMENTAL PROCEDURES

Materials—Iodine-125 radionuclide was purchased from DuPont NEN. Chloramine T hydrate (*N*-chloro-*p*-toluene sulfonamide) and sodium metabisulfite were purchased from Aldrich. Tris and sodium phosphate salts (monobasic and dibasic) were obtained from Calbiochem and Scientific Products/Mallinckrodt, respectively. Hydrochloric

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[1] The abbreviations used are: VEGF, vascular endothelial growth factor; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; CHO, Chinese hamster ovary; RCM, reduced and carboxymethylated; DTT, dithiothreitol; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PlGF, placental growth factor; KDR, kinase domain region; FLT-1, Fms-like tyrosine kinase.

acid, trichloroacetic acid, and Tween 20 (polyethylene-20-sorbitan monolaurate) were from Fisher Scientific. Bovine serum albumin and aprotinin were purchased from Sigma (St. Louis, MO). Gel filtration columns (PD-10), S-Sepharose columns, metal-chelating Sepharose and heparin-Sepharose were from Pharmacia Biotech Inc. Reverse phase and heparin columns were from Vydac (Hesperia, CA) and PerSeptive Biosystems (Cambridge, MA), respectively. Acetonitrile was HPLC-grade from J.T. Baker. The sodium salt of porcine intestinal heparin at 1000 USP units/ml was purchased from Elkins-Sinn, Inc. (Cherry Hill, NJ). Plasmin was from Helena Laboratories (Beaumont, TX). Trypsin, chymotrypsin, Pronase and elastase were from Calbiochem, and collagenase, clostripain, proteinase K, and *Staphylococcus aureus* V8 protease were from Worthington Biochemical (Freehold, NJ). Bromelain, subtilisin, pepsin, and thermolysin were from Boehringer Mannheim. Thrombin was kindly provided by Dr. Walter Kisiel of the University of New Mexico. Affinity-purified rabbit anti-human IgG, Fc specific antibodies and polystyrene 96-well breakaway microtiter plates were purchased from Cappel Laboratories (West Chester, PA) and Dynatech (Chantilly, VA), respectively. Fetal calf serum was from HyClone (Logan, UT). The construction, expression and purification of FLT-1 and KDR receptor-IgG chimeras was as described by Park *et al.* (12).

$VEGF_{165}$ Expressed and Purified from Chinese Hamster Ovary Cells—Recombinant human $VEGF_{165}$ was purified (1) from media conditioned by transfected Chinese hamster ovary cells (7, 13), as described previously. Harvested cell culture fluid was collected after 7 days and concentrated 10-fold by ultrafiltration. The concentrate was diafiltered into 20 mM Na phosphate at pH 7. VEGF was purified by cation exchange chromatography on S-Sepharose and eluted with a gradient of NaCl. Further purification was obtained by metal-chelating chromatography, followed by hydrophobic interaction chromatography using gradient elutions in imidazole and ammonium sulfate, respectively. $VEGF_{165}$ was formulated by gel filtration on G25 Sephadex in 125 mM NaCl and 10 mM sodium citrate at pH 6. Identity was established by SDS-PAGE, reverse phase HPLC, amino-terminal sequencing and amino acid composition.

$VEGF_{165}$ and $VEGF_{121}$ Expressed, Refolded, and Purified from Escherichia coli—We have recently developed a procedure for preparing unglycosylated VEGF from bacterial expression. Transformed *E. coli* cells were lysed by sonication, and the $VEGF_{165}$ (or $VEGF_{121}$) protein was recovered in an insoluble pellet after centrifugation. The pellet was washed with 4 M urea in 20 mM Tris buffer at pH 8 with 5 mM EDTA before solubilization by addition of 25 mM dithiothreitol to the wash buffer. The extraction was allowed to continue for 2 h with stirring at 4 °C before centrifugation to remove insoluble bacterial components. The extract was then dialyzed overnight against 0.4 M NaCl, 20 mM Tris-HCl, pH 8 at 4 °C during which time the extracted protein was allowed to re-fold. The dialyzed, re-folded $VEGF_{165}$ was purified by adsorption to a cation exchange resin (S-Sepharose) and elution with a gradient of 0.4–1.0 M NaCl. Fractions containing dimeric $VEGF_{165}$ (or $VEGF_{121}$), as determined by SDS-PAGE, were pooled and the protein further purified by C4 reverse phase chromatography in 0.1% trifluoroacetic acid with elution by an acetonitrile gradient. $VEGF_{165}$ and $VEGF_{121}$ eluted in approximately 30% acetonitrile. Identity was established by SDS-PAGE under both reducing and non-reducing conditions, by peptide sequencing, amino acid analysis, and mass spectrometry. Refolding of *E. coli*-derived VEGF was evaluated by comparison with CHO-derived $VEGF_{165}$ using monoclonal antibody-based enzyme-linked immunosorbent assay, heparin affinity, and receptor binding as described under "Results."

Enzyme Digests of VEGF—Initial screening of plasmin, thrombin, elastase, collagenase, trypsin, chymotrypsin, pepsin, subtilisin, clostripain, bromelain, Pronase, proteinase K, thermolysin, and *S. aureus* V8 protease for digestion of VEGF was done at enzyme to substrate ratios of 1:100 (by weight) at 25 °C for 24 h at pH 7. Digests were stopped by freezing the samples prior to analysis by reverse phase HPLC and SDS-PAGE under reducing and non-reducing conditions.

Preparation of $VEGF_{165/110}$, $VEGF_{110}$, and the Carboxyl-terminal polypeptide (111–165) from Plasmin Digestion of $VEGF_{165}$—Plasmin was added to purified $VEGF_{165}$ (1:200 ratio by weight) and incubated at 25 °C, pH 7.4, for 4 h for the isolation of $VEGF_{165/110}$ or 24 h for the isolation of $VEGF_{110}$ and carboxyl-terminal polypeptide (111–165). A schematic diagram depicting the plasmin cleavage of VEGF is shown in Fig. 1. The plasmin digests were stopped at the appropriate time by the addition of aprotinin at a 10-fold molar excess with respect to plasmin. Partially digested VEGF (4 h digest) were separated on a POROS heparin HE2 column as described below. The limit digest of VEGF (24 h) was applied to a heparin-Sepharose column (1.5 × 10 cm) pre-equilibrated in 0.15 M NaCl, 50 mM phosphate buffer at pH 7 (PBS).

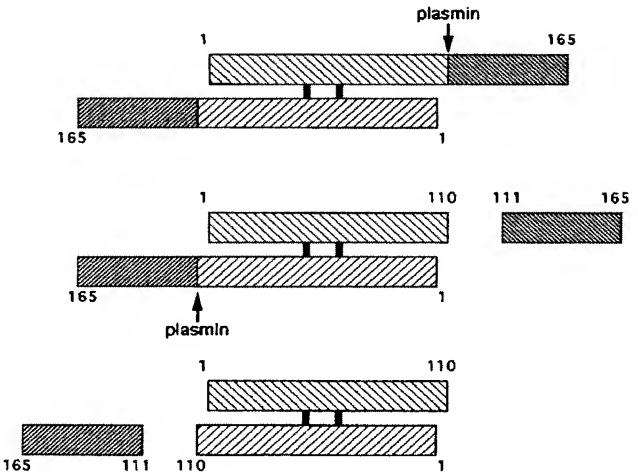

FIG. 1. **Plasmin cleavage of human $VEGF_{165}$.** This schematic diagram depicts the sequential cleavage of the Arg^{110}-Ala^{111} bond in each of the VEGF monomers. The monomers of VEGF are shown in antiparallel orientation, with the carboxyl termini in distant positions, according to homology with the platelet-derived growth factor β dimer crystal structure (35). The monomers of a $VEGF_{165}$ dimer are covalently linked via two interchain disulfide bonds shown as *black bars*. Within the 1–110 and 111–165 domains of each monomer, there are three and four intrachain disulfide bonds, respectively (not shown). There are no interdomain disulfides linking the receptor binding domain (1–110) to the carboxyl-terminal domain (111–165); such that following plasmin cleavage, the 55-amino acid carboxy polypeptides are released from the $VEGF_{110}$ dimer. $VEGF_{165}$, $VEGF_{165/110}$, and $VEGF_{110}$ dimers are shown in the *top*, *middle*, and *bottom*, respectively.

$VEGF_{110}$ eluted from the heparin column in the void volume. After the column was washed (with 10 column volumes of PBS), the carboxyl-terminal polypeptide (111–165) was eluted with 1 M NaCl, 50 mM phosphate at pH 7. The protein containing fractions were dialyzed against PBS. Purity and identity was assessed by SDS-PAGE, amino acid sequence and composition, reverse phase HPLC, and mass spectrometry.

Radiolabeling of VEGF—$VEGF_{165}$ was radiolabeled using a modification of the chloramine T-catalyzed iodination method described by Hunter and Greenwood (14). In a typical reaction, 50 μl of 1 M Tris-HCl, 0.01% Tween 20 at pH 7.5 was added to 5 μl of sodium iodide-125 (0.5 mCi) in a capped reaction vessel. An aliquot of VEGF (10 μg/10 μl) in 125 mM NaCl, 10 mM sodium citrate at pH 6 was added to the reaction vessel. Iodination was initiated by addition of 12.5 μl of 1 mg/ml chloramine T in 0.1 M sodium phosphate, pH 7.5. After 60 s, iodination was terminated by addition of sodium metabisulfite (25 μl, 1 mg/ml) in 0.1 M sodium phosphate, pH 7.5. The reaction vessel was vortexed after each addition. The reaction mixture was immediately applied to a PD-10 column (G25 Sephadex) that was pre-equilibrated with 0.5% bovine serum albumin, 0.01% Tween 20 in phosphate-buffered saline. Fractions were collected and counted for iodine-125 radioactivity with a γ scintillation counter (LKB model 1277). Typically, the specific radioactivity of the iodinated VEGF was 26 ± 2.5 μCi/μg, which corresponded to approximately 1 $^{125}I/2$ molecules of $VEGF_{165}$ dimer. There are four tyrosines in $VEGF_{165}$ at positions 21, 25, 39, and 45. Tryptic mapping of RCM $VEGF_{165}$ indicated approximately 30% and 70% of the radioactivity co-eluted with tryptic peptides T2 (amino acids 17–23) and T3 (amino acids 24–56), respectively.

Dodecyl Sulfate Gel Electrophoresis—Radiolabeled samples were either reduced, or reduced and carboxymethylated (RCM) prior to gel electrophoresis. The reduced samples were denatured in 1% SDS with 10 mM dithiothreitol (DTT) and heated at 37 °C for 30 min. Samples for reduction and carboxymethylation were treated by a modified procedure of Crestfield *et al.* (15). Those samples were dialyzed overnight in 8 M urea, 0.5 M Tris-HCl at pH 8.3 with 5 mM EDTA, then reduced with 10 mM DTT at 37 °C for 30 min. Iodoacetic acid (1 M IAA in 1 M NaOH) was added to a final concentration of 25 mM and incubated at 25 °C for 15 min. The alkylation reaction was quenched by addition of 25 mM DTT, followed by overnight dialysis at 4 °C with 50 mM NH_4CO_3. Electrophoretic analysis was by the method of Laemmli (16) on a SDS

gradient acrylamide gel (10–20%), followed by either Coomassie Blue or ammoniacal silver staining (17). After the stained gels were dried, the radiolabeled proteins were visualized by autoradiography.

Amino Acid Analysis and Ultraviolet Spectroscopy—A Beckman 6300 amino acid analyzer was used with a sodium citrate program and ninhydrin detection. Aliquots (10 μl each) of VEGF samples were hydrolyzed in constant boiling HCl for 24 h at 110 °C. Quantitation was based on the yields of alanine and leucine. VEGF samples were diluted in 125 mM NaCl, 10 mM sodium citrate at pH 6 and scanned for UV absorption from 190 to 800 nm on a Hewlett Packard 8452A diode array spectrophotometer. The molar extinction coefficient for $VEGF_{165}$ was determined as 0.37 absorbance units for a 1 mg/ml solution at 276 nm.

Mass Spectrometry—The molecular weights of VEGF variants were analyzed using a Sciex API III triple quadrupole mass spectrometer. The data was obtained by scanning from 300 to 2000 Da with a 0.8-ms dwell time per mass step. A Harvard infusion pump was used to introduce the samples into the mass spectrometer at a flow rate of 3–5 μl/min. The data was collected in the data summing mode and analyzed using MacBioSpec software.

Reverse Phase HPLC—Analytical separations of VEGF fragments were done using a Vydac C_4, 5-μm bead, 300-Å pore size column (4.6 × 250 mm) pre-equilibrated in 0.1% trifluoroacetic acid, 5% acetonitrile at 40 °C on a Hewlett Packard 1090 liquid chromatograph with diode array detection. Typically, an aliquot of VEGF (10 μl) containing 30 μg was injected onto the HPLC column with a flow rate of 1 ml/min. Reverse phase separation of VEGF and VEGF fragments was done with a two-step gradient from 5% to 25% acetonitrile with 0.1% trifluoroacetic acid for 20 min, followed by a shallow gradient from 25% to 33% in 32 min. The effluent was monitored for absorbance (at 210 and 280 nm).

Heparin Affinity Chromatography—Analytical and preparative affinity chomatography was performed on a HP1090 liquid chromatograph using a POROS HE2 heparin column (4.6 × 100 mm). The column was pre-equilibrated with 50 mM Na_2PO_4 buffer at pH 7.4 using a 0.5 ml/min flow rate at 40 °C. Samples of VEGF were injected and eluted with a salt gradient from 0 to 1 M NaCl in 40 min (25 mM/min). The effluent was monitored for absorbance at 210 and 280 nm. Fractions (0.5 ml each) were collected and further analyzed as described under "Results."

Binding Assays with Soluble Receptors—Polystyrene 96-well breakaway microtiter plates were coated overnight at 4 °C with 100 μl of affinity-purified rabbit anti-human IgG, Fc-specific antibodies at 10 μg/ml in 50 mM Na_2CO_3 at pH 9.6. The microtiter plates were blocked with 200 μl of 10% fetal calf serum in phosphate-buffered saline (FBS/PBS) for 1 h at 25 °C. Blocking buffer was removed, and 100 μl of a solution containing receptor-IgG chimeric protein (FLT1-IgG or KDR-IgG) at 15 ng/ml (70 pM final concentration), ^{125}I labeled $VEGF_{165}$ from CHO cells (5000 cpm per well, 20 pM final), and cold competitor at varying concentrations in PBS with 10% FBS was added to the microtiter wells. Binding was carried out at ambient temperature for 4 h with gentle agitation, after which the wells were washed four times with 10% FBS in PBS. The bound radioactivity was quantitated with a γ scintillation counter (LKB model 1277). Binding data was analyzed using a four-parameter fitting program (Kaleidagraph, Adelbeck Software). The receptor binding studies were repeated in the presence of heparin (10 μg/ml) to observe the maximal effect of heparin on the receptor-VEGF interaction. In these studies, we have used heparin as a commercial reagent to help address the biologic significance of the various forms of VEGF. Similar to the results observed by Tessler *et al.* (18) and Gitay-Goren *et al.* (26), dose-response studies with increasing heparin indicated the optimal concentration to achieve maximal KDR receptor binding.

Endothelial Cell Proliferation Assay for VEGF—Bovine adrenal cortical capillary endothelial cells were maintained in low glucose Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 2 mM glutamine, and antibiotics (growth medium) as described previously (1). For bioassay, cells were sparsely seeded in 12-well plates with 7×10^3 cells/well in 1 ml of growth medium. Samples of VEGF or VEGF variants (1 ml) were diluted in the culture media at concentrations from 1 μg/ml to 10 pg/ml (final) and layered onto the seeded cells. After 5 days, the cells were dissociated with trypsin and quantified using a Coulter Counter (Miami, FL).

RESULTS

Limited Proteolysis of VEGF—The susceptibility of VEGF to proteolytic digestion was explored using the glycosylated form of $VEGF_{165}$ as expressed and purified from CHO cell conditioned media. As observed in Fig. 2*A* (*lane 2*), $VEGF_{165}$ appeared on SDS-PAGE as a doublet of proteins at 43 and 45 kDa. Tryptic mapping of reduced and carboxymethylated VEGF indicated approximately 75% of the protein contained *N*-linked glycosylation at Asn^{75}; the remaining 25% of VEGF was unglycosylated (data not shown). The doublet of protein observed on SDS-PAGE was due to partial glycosylation. As seen in other studies, $VEGF_{165}$ from *E. coli* appeared as a single protein band of 38 kDa. A variety of enzyme digests of $VEGF_{165}$ were prepared under similar conditions (1:100, enzyme:VEGF at 25 °C and for 20 h). Relatively complete digestion was observed on reverse phase HPLC with broad-specificity enzymes, such as subtilisin, Pronase, proteinase K, and thermolysin. Enzymes such as plasmin, trypsin, chymotrypsin, clostripain, and bromelain yielded varying extents of partial digestion resulting in a "core protein," which was resistant to further proteolysis. No proteolysis was observed with thrombin, collagenase, elastase, *S. aureus* V8 protease, or pepsin as indicated by HPLC. The digestion profiles were similar for CHO- and *E. coli*-derived $VEGF_{165}$. A series of enzyme digests were analyzed by non-reducing SDS-PAGE, which indicated that trypsin and plasmin cleaved VEGF, but thrombin, elastase, chymotrypsin, or pepsin did not (Fig. 2*A*). These enzyme digests were reduced, carboxymethylated, and evaluated by SDS-PAGE (Fig. 2*B*) to observe additional proteolysis that was not apparent under non-reducing conditons. Some proteolysis of VEGF by chymo-

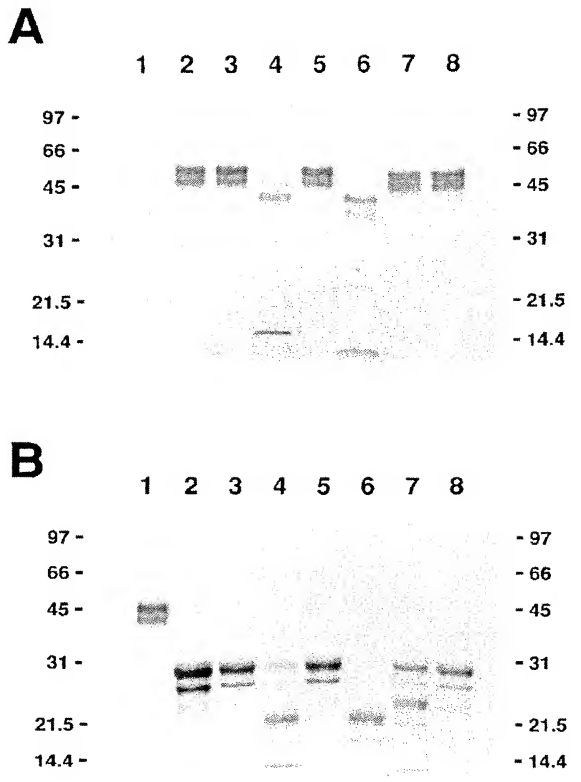

FIG. 2. **Enzymatic digestion of CHO-derived $VEGF_{165}$.** Digests with thrombin, plasmin, elastase, trypsin, chymotrypsin, and pepsin are evaluated in *panel A*, by non-reducing SDS-PAGE. In *panel A*, *lanes 1*, blank; *lane 2*, non-reduced CHO-derived $VEGF_{165}$; *lane 3*, thrombin; *lane 4*, plasmin; *lane 5*, elastase; *lane 6*, trypsin; *lane 7*, chymotrypsin; *lane 8*, pepsin. In *panel B*, the samples were RCM after the enzyme digestion. *Lane 1*, non-reduced CHO-derived $VEGF_{165}$; *lane 2*, RCM CHO-derived $VEGF_{165}$; *lane 3*, thrombin; *lane 4*, plasmin; *lane 5*, elastase; *lane 6*, trypsin; *lane 7*, chymotrypsin; *lane 8*, pepsin digests of RCM CHO-derived $VEGF_{165}$.

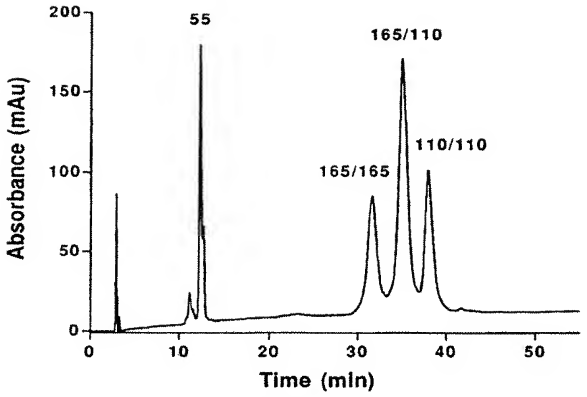

FIG. 3. **Reverse phase HPLC separation of plasmin digest products of *E. coli*-derived $VEGF_{165}$.** After 4 h of proteolysis at 25 °C with plasmin (1:200, enzyme:substrate), the partial digest products were resolved by HPLC. The plasmin digest products include: the 55-amino acid carboxyl-terminal polypeptide (*55*), $VEGF_{165}$ homodimer (*165/165*), $VEGF_{165/110}$ heterodimer (*165/110*), $VEGF_{110}$ homodimer (*110/110*), which eluted at 12, 32, 35, and 38 min, respectively.

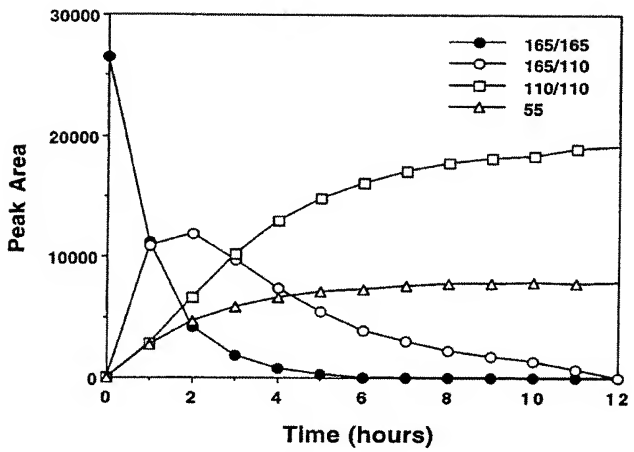

FIG. 4. **Kinetics of plasmin cleavage of $VEGF_{165}$.** The progress of plasmin digestion was followed by repetitive reverse phase HPLC analysis at hourly intervals. The HPLC eluate was monitored for absorbance at 210 nm, and the amount of $VEGF_{165}$ homodimer, $VEGF_{165/110}$ heterodimer, $VEGF_{110}$ homodimer, and the 55-amino acid carboxyl-terminal polypeptide at the indicated times were determined by peak area integration.

trypsin was observed (*lane 7*), in addition to the cleavage of VEGF by plasmin and trypsin (*lanes 4* and *6*). $VEGF_{165}$ is relatively resistant to proteolysis by enzymes such as collagenase, thrombin, and elastase. However, plasmin rapidly cleaves VEGF into discrete, non-disulfide-linked fragments.

Isolation and Identification of Plasmin-cleaved VEGF Fragments—To eliminate the apparent heterogeneity due to glycosylation of CHO cell expressed $VEGF_{165}$, we studied the activity of plasmin on *E. coli*-derived $VEGF_{165}$. The absence of carbohydrate on this form of VEGF was confirmed by tryptic mapping. A plasmin digest of $VEGF_{165}$ from *E. coli* (1:200 enzyme:substrate) was initiated and samples removed at various times for analysis by reverse phase HPLC. After 2 h of plasmin treatment, four protein peaks with absorption at 210 nm were resolved by HPLC (Fig. 3). The first peak, eluting at 12 min, exhibited minimal absorption at 280 nm, consistent with the carboxyl-terminal region of VEGF, which has only one aromatic amino acid, phenylalanine 128. Protein containing HPLC fractions were collected, pooled and identified by amino acid sequence, composition, and mass spectrometry. The early eluting peak of plasmin-cleaved $VEGF_{165}$ was identified as the polypeptide (111–165) by the amino-terminal sequence: Ala^{111}-Arg-Gln-Glu-Asn-Pro^{116}. The amino acid composition and minimal retention on reverse phase HPLC was consistent with the highly charged, hydrophilic 55-amino acid, carboxyl-terminal polypeptide. Mass spectral analysis indicated a molecular weight of 6473 atomic mass units for the (111–165) polypeptide, which compared well with the expected value of 6474 atomic mass units based on the amino acid sequence. The later eluting HPLC peaks, with retention times of 32, 35, and 38 min were identified as homodimer of (1–165), heterodimer of (1–165, 1–110) and homodimer of (1–110), respectively. All of the later eluting peaks shared the amino-terminal sequence: Ala^{1}-Pro-Met-Ala-Glu^{5}. The homodimer of (1–165) and the homodimer of (1–110) exhibited molecular weights of 38,306 and 25,385 atomic mass units, which compared well with the theoretical values of 38,300 and 25,389 atomic mass units, respectively. Retention times of these plasmin cleavage products are consistent with the loss of the hydrophilic carboxyl-terminal polypeptide (111–165), such that the heterodimer (1–165, 1–110) and homodimer (1–110) are progressively more hydrophobic and later eluting on reverse phase HPLC.

Kinetic Analysis of Plasmin Cleavage—Fig. 4 shows the kinetics for plasmin cleavage of *E. coli*-derived $VEGF_{165}$ (enzyme:substrate 1:100) at pH 7. The digest was analyzed at various times by reverse phase HPLC to quantify the amount of each form of VEGF. Cleavage of intact homodimer (1–165) occurred with a concomitant increase in the heterodimer (1–165, 1–110) followed by the appearance of the homodimer (1–110). The amount of (111–165) polypeptide increased over time and achieved a maximal level, which was approximately 40% of that observed for the (1–110) homodimer. Additional proteolysis of the cleaved polypeptide (111–165) by plasmin leads to lower molecular weight species, which eluted at 11 min on HPLC (Fig. 3). The plasmin catalyzed cleavage of $VEGF_{165}$ homodimer was approximately 3-fold faster than that observed for $VEGF_{165/110}$ cleavage, as indicated by the rate of disappearance for each protein observed on HPLC. The observation that $VEGF_{165}$ homodimer was cleaved faster than the $VEGF_{165/110}$ heterodimer was unexpected, since the two plasmin cleavage sites (Arg^{110}-Ala^{111} in each monomer) are identical in the dimer. We considered that the lysine-rich polypeptide (111–165) of VEGF may act as an additional recognition site for plasmin on VEGF and that the kringles of plasmin (kringles 1, 4, and 5) may function as lysine-binding modules for binding VEGF. Fig. 1 shows schematically the sequential cleavage of VEGF by plasmin. In the first cleavage, the intact carboxyl-terminal region of VEGF presented by the opposing $VEGF_{165}$ monomer may increase binding and cleavage of the full-length monomer, but does not participate in the binding of the heterodimer resulting in reduced rate for the second cleavage of $VEGF_{165/110}$.

Heparin Binding of Homo- and Heterodimeric VEGF—The heparin binding function of $VEGF_{165}$ was studied by analytical affinity chromatography. Samples of *E. coli*-derived $VEGF_{165}$, before and after treatment with plasmin, were applied to a POROS heparin column and eluted with an increasing gradient of NaCl. The eluate was monitored for optical density at 210 nm, and appropriate pooled fractions were analyzed by reverse phase HPLC. The results, shown in Table I, indicate that the heparin binding function of $VEGF_{165}$ is completely mediated by the carboxyl-terminal domain (111–165). The heparin affinity of $VEGF_{165}$ and the polypeptide (111–165) are nearly equivalent as determined by the concentration of NaCl required for elution (680 and 690 mM, respectively). Heterodimeric

TABLE I
Relative heparin affinity of VEGF fragments as indicated by the ionic strength required for elution from heparin

Identity	Retention time	NaCl	Percent of control[a]
	min	*mM*	
$VEGF_{165}$ homodimer	27.2	680	100
$VEGF_{165/110}$ heterodimer	16.7	420	62
$VEGF_{110}$ homodimer	2.5	60	9
$VEGF_{121}$ homodimer	2.5	60	9
55-amino acid polypeptide	27.5	690	101

[a] Percent of control describes the difference in heparin affinity as a function of the salt concentration required to elute the sample. The control for each experiment was the VEGF used as starting material for the plasmin digest.

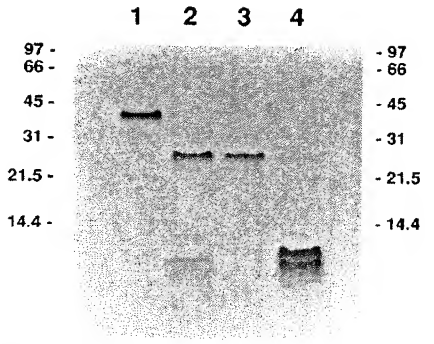

FIG. 5. **SDS-PAGE of plasmin digested *E. coli*-derived $VEGF_{165}$.** *Lane 1*, native *E. coli*-derived $VEGF_{165}$; *lane 2*, plasmin digest at 20 h; *lane 3*, heparin-Sepharose column flow-though containing $VEGF_{110}$ homodimer; *lane 4*, 1 M NaCl eluate containing predominantly (111–165) polypeptide, and trace amounts of $VEGF_{165}$ homodimer, $VEGF_{165/110}$ heterodimer, and $VEGF_{110}$ homodimer.

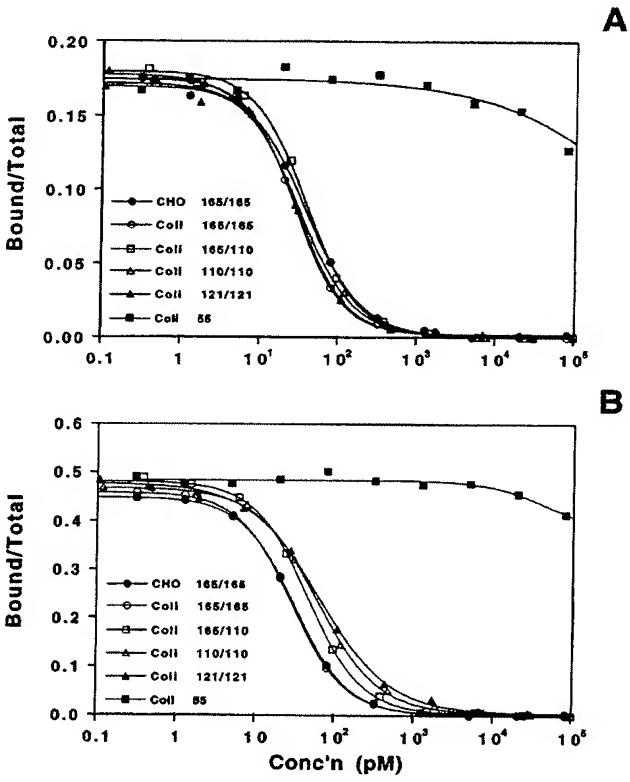

FIG. 6. **Binding of VEGF isoforms to soluble KDR-IgG receptor.** The competitive displacement of ^{125}I-labeled CHO-derived $VEGF_{165}$ binding to KDR receptor (15 ng/ml) with various concentrations of CHO-derived $VEGF_{165}$ (●), *E. coli*-derived $VEGF_{165}$ (○), *E. coli*-derived $VEGF_{165/110}$ heterodimer (□), $VEGF_{110}$ homodimer (△), $VEGF_{121}$ homodimer (▲), and 55-amino acid carboxyl-terminal polypeptide (■). *Panels A* and *B* show the results of the binding studies in the absence and presence of heparin, respectively. These binding curves are the result of triplicate assays.

$VEGF_{165/110}$ eluted at 420 mM NaCl, indicating a significant decrease in heparin affinity associated with the loss of one carboxyl-terminal domain. No heparin binding was observed for homodimeric $VEGF_{110}$ and $VEGF_{121}$. Various conditions for plasmin digestion were used to prepare hetero- and homodimeric VEGF, followed by preparative heparin chromatography. Fig. 5 shows *E. coli* $VEGF_{165}$, lacking carbohydrate modification, which yields a single band on SDS-PAGE with an apparent molecular mass of 38 kDa (*lane 1*). The SDS-PAGE analysis of a plasmin digest of *E. coli* $VEGF_{165}$ appears in *lane 2*. $VEGF_{110}$ homodimer was not retained on heparin-agarose (*lane 3*), while the (111–165) polypeptide with trace amounts of homo- and heterodimeric VEGF were eluted with 1 M NaCl (*lane 4*). Gradient elution of partial plasmin digests on a PORO S-heparin column yielded highly purified preparations of $VEGF_{165/110}$ and $VEGF_{110}$ as indicated by reverse phase HPLC (data not shown).

Binding of VEGF Isoforms to Soluble Receptors in the Presence and Absence of Heparin—Binding of radioiodinated CHO-derived $VEGF_{165}$ to KDR-IgG and FLT1-IgG was analyzed using a competitive displacement radioreceptor assay (Figs. 6 and 7). Various concentrations of unglycosylated (expressed in *E. coli*) $VEGF_{165}$, $VEGF_{165/110}$, $VEGF_{110}$, and $VEGF_{121}$ were tested for displacement of labeled glycosylated (expressed in CHO cells) $VEGF_{165}$. The 55-amino acid, carboxyl-terminal domain of VEGF (111–165) was tested at 1000-fold molar excess and did not inhibit VEGF binding to the soluble receptors. At concentrations greater than 1000-fold molar excess, the isolated carboxyl-terminal domain appeared to partially displace $VEGF_{165}$ from receptor. Radiolabeled VEGF binding to the soluble form of KDR was half-maximally displaced at 38 pM $VEGF_{165}$ in the absence of heparin (Fig. 6*A*). Glycosylation of $VEGF_{165}$ did not affect KDR binding, as indicated by the similar affinity exhibited by CHO- and *E. coli*-derived $VEGF_{165}$ (Table II). Loss of one or both carboxyl-terminal domain(s) had no effect on the affinity of VEGF for KDR as indicated by the IC_{50} values for $VEGF_{165/110}$ heterodimer and $VEGF_{110}$ and $VEGF_{121}$ homodimers (38, 29, and 30 pM, respectively). In the presence of heparin, the amount of VEGF bound to KDR was increased more than 3-fold; however, the apparent affinity of VEGF for KDR was unchanged (Fig. 6*B*). Glycosylated and unglycosylated $VEGF_{165}$ bound KDR with equivalent IC_{50} values (31 and 28 pM, respectively). Loss of carboxyl-terminal domains resulted in 2-fold decreased KDR affinity (in the presence of heparin), as observed in competition studies with $VEGF_{165/110}$ heterodimer and $VEGF_{110}$ and $VEGF_{121}$ homodimers (IC_{50} values of 45, 60, and 63 pM, respectively).

Glycosylated and unglycosylated $VEGF_{165}$ exhibited similar affinity for soluble FLT-1 receptor, approximately 10 pM in the absence of heparin (Fig. 7*A*). Loss of one carboxyl-terminal domain was associated with 3-fold decreased FLT-1 affinity as indicated by the binding of $VEGF_{165/110}$ heterodimer. The loss of both carboxyl-terminal domains resulted in approximately 10-fold reduced FLT-1 affinity as exhibited by the $VEGF_{110}$ homodimer. Similarly, the values obtained for the natural splice variant $VEGF_{121}$ indicated greater than 20-fold decreased binding to FLT-1 compared with that observed for $VEGF_{165}$ (Table II). The carboxyl-terminal domain itself

(polypeptide 111–165) exhibited no binding to FLT-1 (as was observed with KDR). In the presence of heparin, the differences in FLT-1 binding observed for the VEGF variants were diminished (Fig. 7*B*). Glycosylated and unglycosylated VEGF 165 bound soluble FLT-1 with values for IC_{50} of 15 and 19 pM, respectively. $VEGF_{165/110}$ also exhibited similar binding affinity. The binding values for $VEGF_{121}$ and $VEGF_{110}$ were reduced approximately 2–3-fold, respectively, compared to those observed for $VEGF_{165}$.

Differential Stimulation of Endothelial Cell Growth Induced by VEGF Isoforms—Stimulation of endothelial cell proliferation by VEGF variants was evaluated with bovine adrenal cortical capillary endothelial cells (Fig. 8). The effective concentration to induce 50% of maximal stimulation (EC_{50}) was determined *in vitro* by incubation of endothelial cells with VEGF or VEGF variants at concentrations that varied from 0.3 pM to 40 μM. Glycosylated and unglycosylated $VEGF_{165}$ exhibited similar EC_{50} values of 5.8 and 5.2 pM, respectively. The loss of one carboxyl-terminal domain resulted in approximately 7-fold decrease in potency for $VEGF_{165/110}$ as indicated by the higher EC_{50} value of 40 pM. Lower molecular weight variants, $VEGF_{110}$ and $VEGF_{121}$, displayed greater than 100-fold reduced potency with values of 2.58 and 2.56 nM for EC_{50}, respectively. The isolated carboxyl-terminal domain (111–165) had no stimulatory effect on endothelial cell proliferation even with 4 orders of magnitude molar excess of polypeptide compared to the half-maximally effective concentration for $VEGF_{165}$. Similar results were observed with fetal bovine aortic endothelial cells with respect to a 10-fold and 100-fold loss in mitogenic potency with $VEGF_{165/110}$ and $VEGF_{110}$ (or $VEGF_{121}$), respectively (data not shown). These results demonstrate the critical role of the 111–165 region of VEGF in the stimulation of both large and small vessel endothelial cell proliferation.

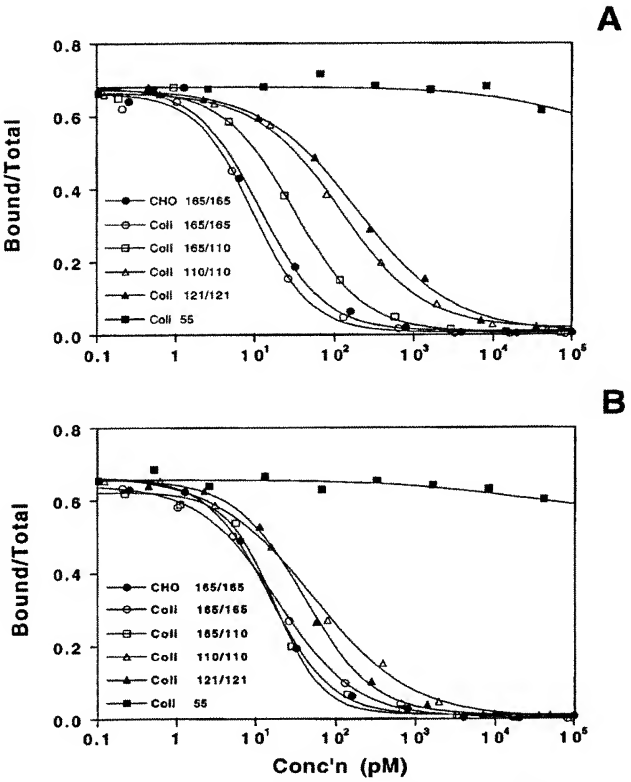

FIG. 7. **Binding of VEGF isoforms to soluble FLT-1 IgG receptor.** The competitive displacement of ^{125}I-labeled CHO-derived $VEGF_{165}$ binding to FLT-1 receptor (5 ng/ml) with various concentrations of CHO-derived $VEGF_{165}$ (●), *E. coli*-derived $VEGF_{165}$ (○), *E. coli*-derived $VEGF_{165/110}$ heterodimer (□), $VEGF_{110}$ homodimer (△), $VEGF_{121}$ homodimer (▲), and 55-amino acid carboxyl-terminal polypeptide (■). *Panels A* and *B* show the results of the binding studies in the absence and presence of heparin, respectively. These binding curves are the result of duplicate assays.

DISCUSSION

Extracellular proteolysis and remodeling of the extracellular matrix play key roles in a variety of developmental processes (19). In addition, plasminogen activation and generation of plasmin have been shown to be important for the angiogenesis cascade (20). Such processes also play a major role in the local invasiveness and metastasis of tumor cells. Strong experimental evidence supports the concept that growth factors stored in the extracellular matrix and released in the course of its degradation are major mediators of such inductive processes. Numerous growth factors including fibroblast growth factor, platelet-derived growth factor, granulocyte/macrophage colony-stimulating factor, transforming growth factor-β, and leukemia inhibitory factor have been shown to be associated with the extracellular matrix (21–25).

Alternatively spliced molecular species of VEGF are differentially localized to heparan sulfate containing proteoglycans of the extracellular matrix or released as diffusible proteins (10, 11). In addition to this transcriptionally regulated heterogeneity, there appears the potential for proteolytic processing which may further regulate the bioavailability of VEGF. In the present study, we have demonstrated that plasmin readily cleaves $VEGF_{165}$ to yield a non-heparin binding isoform, $VEGF_{110}$, and a heparin-binding fragment composed of the carboxyl-terminal domain (111–165). In contrast to plasmin, neither thrombin, elastase, nor collagenase efficiently cleaves VEGF. Like $VEGF_{121}$, $VEGF_{110}$ has no affinity for heparin and is a diffusible molecule (11). Therefore, the carboxyl-terminal domain (amino acids 111–165) is completely responsible for the observed heparin binding of VEGF *in vitro*. Interestingly, a partially cleaved form, $VEGF_{165/110}$ was isolated that exhibited

TABLE II
Receptor binding affinity for VEGF fragments (IC_{50})

IC_{50} represents the concentration of inhibitor (pM) required to achieve half-maximal binding of labeled, glycosylated $VEGF_{165}$. Duplicate or triplicate analyses were performed and coefficients of variation were less than 10% for all values of IC_{50} except for FLT-IgG binding determinations in the absence of heparin (i.e., average cv = 30%).

Sample	KDR-IgG		Ratio[a]	FLT-IgG		Ratio[a]
	+ heparin	− heparin		+ heparin	− heparin	
CHO 165/165	31 ± 2	38 ± 5	0.8	15 ± 1	12 ± 3	1.25
E. coli 165/165	28 ± 0.3	27 ± 1	1.0	19 ± 2	10 ± 2	1.90
E. coli 165/110	45 ± 0.5	38 ± 7	1.2	17 ± 3	31 ± 11	0.55
E. coli 110/110	60 ± 5	29 ± 0.2	2.1	51 ± 8	120 ± 45	0.43
E. coli 121/121	63 ± 1	30 ± 1	2.1	40 ± 1	195 ± 80	0.21

[a] Ratio of the apparent K_d for the VEGF samples with each soluble receptor in the presence of heparin versus the absence of heparin, i.e. IC_{50} (+ heparin)/IC_{50} (− heparin). The concentration of added heparin was 10 μg/ml.

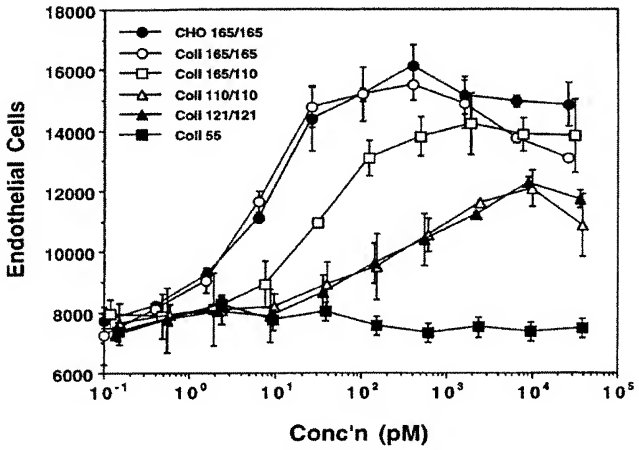

FIG. 8. **Endothelial cell proliferation in response to VEGF isoforms.** Bovine adrenal cortical endothelial cells were cultured for 5 days in the presence of varying concentrations of CHO-derived $VEGF_{165}$ (●), *E. coli*-derived $VEGF_{165}$ (○), *E. coli*-derived $VEGF_{165/110}$ heterodimer (□), $VEGF_{110}$ homodimer (△), $VEGF_{121}$ homodimer (▲), and 55-amino acid carboxyl-terminal polypeptide (■). The cells were trypsinized and counted as described under "Experimental Procedures." These results represent the average of duplicate assays.

intermediate heparin affinity.

We have used the multiple forms of VEGF obtained by plasmin cleavage or alternative splicing to evaluate the role of the carboxyl-terminal domain on VEGF receptor affinity and endothelial cell growth. The 55-amino acid peptide (111–165) of VEGF did not bind the soluble KDR receptor, as indicated by the complete lack of inhibition observed with ^{125}I-labeled $VEGF_{165}$ binding. Binding to the KDR receptor is mediated by determinants in the 1–110 region of $VEGF_{165}$. The lower molecular weight diffusible forms, $VEGF_{110}$ and $VEGF_{121}$, bind KDR with similar affinity as $VEGF_{165}$ in the absence of heparin. A modest effect of heparin is the 3–4-fold increased capacity of KDR for binding VEGF. The heparin-induced potentiation of VEGF binding to KDR has also been observed by other investigators. Gitay-Goren *et al.* (26) observed an increased number of high affinity VEGF binding sites on endothelial cells in the presence *versus* the absence of heparin, but heparin did not significantly alter the dissocation constant. Tessler *et al.* (18) also reported increased binding of $VEGF_{165}$ to Flk-1/KDR type receptors transfected into NIH3T3 cells as a function of heparin.

FLT-1 binding is significantly different for the long and short forms of VEGF. In contrast to the results observed with KDR, the shorter forms of VEGF (110 and 121 forms) bound FLT-1 receptor with 10–20-fold decreased affinity compared to $VEGF_{165}$. $VEGF_{165/110}$ displayed FLT-1 binding intermediate to the 165 and 110 forms of VEGF. Taken together, these results demonstrate that the 55-amino acid domain of VEGF mediates, in part, the binding to FLT-1. This domain appears to enhance FLT-1 binding in addition to the receptor binding determinants contained in $VEGF_{110}$, but by itself the 55-amino acid domain cannot compete with $VEGF_{165}$ binding to FLT-1, as indicated by the lack of competition with greater than 1000-fold molar excess of (111–165) polypeptide (see Fig. 7). As such, the 55-amino acid, carboxyl-terminal domain of $VEGF_{165}$ plays a different role with KDR as compared to FLT-1 in mediating receptor binding. For example, there is no heparin-induced potentiation of FLT-1 binding of VEGF as is observed with KDR. Cohen *et al.* (27) have observed the inhibition of heparin on VEGF binding to FLT-1 receptors on human melanoma cells. These investigators observed significantly decreased melanoma cell binding of ^{125}I-labeled $VEGF_{121}$ and $VEGF_{165}$ in the presence of exogenous heparin (1 μg/ml) compared to that observed in the absence of heparin.

VEGF interaction with cell surface heparan-sulfate containing proteoglycans on endothelial cell growth has been examined by numerous investigators (18, 25–30). Sasisekharan *et al.* (30) have indicated a role for heparin-like molecules by inhibiting endothelial cell proliferation and *in vivo* neovascularization with heparinase. The binding of ^{125}I-labeled $VEGF_{165}$ to endothelial cells was completely inhibited by pretreatment with heparinase and could be restored by the addition of exogenous heparin (25). To evaluate the significance of the relative heparin affinity of VEGF isoforms, we tested the mitogenic activity of the 165, 165/110, 110, and 121 forms of VEGF on primary cultured bovine adrenal cortical capillary cells and fetal bovine aortic endothelial cells. Loss of one or both carboxyl-terminal domains of VEGF significantly reduced the proliferation of endothelial cells. The mitogenic potency of $VEGF_{110}$ and $VEGF_{121}$ was substantially decreased (>100-fold) compared to $VEGF_{165}$. $VEGF_{165/110}$ exhibited 7–10-fold reduced activity on endothelial cell growth. These results are most interesting in light of the similarities and differences observed with various VEGF isoforms binding soluble KDR or FLT-1 receptors. The modestly decreased affinity observed with soluble KDR and FLT-1 receptors and the 110 or 121 isoforms of VEGF does not account for the drastically decreased endothelial cell mitogenic potency. This discrepancy suggests that the stability of VEGF-heparan sulfate-receptor complexes probably contributes to effective signal transduction and stimulation of endothelial cell proliferation. Further studies are clearly warranted to explore the regulatory function of heparin-like molecules in VEGF receptor interaction, signal transduction, and mitogenesis of endothelial cells.

Our findings indicate that VEGF, by alternative splicing and/or limited proteolysis, has the potential to express structural and functional heterogeneity that yields a graded biological response. Our current understanding of VEGF biology suggests the following sequence of events may occur during angiogenesis. Initially, cells respond to hypoxia or other stimuli by inducing VEGF transcription (31), resulting in increased expression of long and short forms of VEGF, although $VEGF_{165}$ is probably the most abundant isoform. Compared to the longer forms of VEGF, the diffusible forms would migrate a greater distance, bind VEGF receptors, and trigger endothelial cell proliferation and migration. The intensity of the angiogenic signal would be weakest at the most distant sites, given the lesser mitogenic potency of $VEGF_{110}$ and $VEGF_{121}$. Closer to the site of VEGF synthesis (*i.e.* ischemic tissues), the concentration of the 165 isoform is expected to be increased due to extracellular matrix binding and the effect of the biochemical gradient would be enhanced with the associated greater mitogenic potency of $VEGF_{165}$. In the most ischemic areas, matrix-associated VEGF is localized to the cells of origin with the highest concentration and potency. The heterogeneity of VEGF structure and function allows the formation of a biochemical gradient for the migration and chemotaxis of proliferating endothelial cells. In circumstances where plasminogen activation occurs (*e.g.* tumors and wounds), the presence of plasmin may serve to release stored forms of matrix-bound VEGF to amplify the angiogenic signal.

Placental growth factor (PlGF) is a protein with 53% homology to VEGF (32, 33). PlGF binds FLT-1 with similar affinity as VEGF, but does not bind to the KDR receptor (12). Interestingly, recent studies describing the existence of VEGF·PlGF heterodimers further extend the concept that structural heter-

ogeneity may be responsible for a graded biological response. While the PlGF homodimer had minimal endothelial cell mitogenic activity compared to that of the VEGF homodimer, the heterodimer displayed intermediate activity (34). With respect to proteolytic digestion resulting in the generation of $VEGF_{110}$ from $VEGF_{165}$ or $VEGF_{189}$, little is known at this time about the prevalence of this process *in vivo*. Nevertheless, the potential exists for VEGF to set up a biochemical gradient that radiates from an ischemic zone and may provide a directional signal for *in vivo* angiogenesis.

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